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Characterization of the *Vibrio cholerae* El Tor Lipase Operon *lipAB* and a Protease Gene Downstream of the *hly* Region

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We have cloned and sequenced a region encoding a lipase operon and a putative, previously uncharacterized metalloprotease of *Vibrio cholerae* O1. These lie downstream of *hlyA* and *hlyB*, which encode the El Tor hemolysin and methyl-accepting chemotactic factor, respectively. Previous reports identified the *hlyC* gene downstream of *hlyAB*, encoding an 18.3-kDa protein. However, we now show that this open reading frame (ORF) encodes a 33-kDa protein, and since the amino acid sequence is highly homologous to the triacylglyceride-specific lipase of *Pseudomonas* spp., *hlyC* has been renamed *lipA*. LipA contains the highly conserved pentapeptide and catalytic triad amino acid regions of the catalytic sites of other lipases. The region downstream of *lipA* has been sequenced and has revealed ORFs *lipB* and *prtV*. The amino acid sequence of *lipB* is homologous to those of the accessory lipase proteins (lipase-specific foldase) required by *Pseudomonas* and various other bacterial species for the production of mature active lipase, and in agreement with this, we show that both *lipA* and *lipB* are required to restore a lipase-deficient *lipA* null mutant of *V. cholerae*. The intergenic stop codon for *lipA* overlaps the ribosome-binding site for *lipB*, and a stem-loop resembling a rho-independent terminator is present immediately downstream from *lipB*, suggesting that *lipA* and *lipB* form a lipase operon in *V. cholerae*. *prtV* lies downstream of *lipAB* but is transcribed in the opposite direction and is predicted to share the same putative transcriptional terminator with *lipAB*. The zinc-binding and catalytic domains conserved among many metalloproteases are present in PrtV, which is highly homologous to the immune inhibitor A (InA) metalloprotease of *Bacillus thuringiensis*. PrtV was visualized as approximately 102 kDa, which is consistent with the coding capacity of the gene. The genetic organization of this region suggests that it is possibly part of a pathogenicity island, encoding products capable of damaging host cells and/or involved in nutrient acquisition by *V. cholerae*. However, neither *lipA* nor *prtV* null mutants were attenuated in the infant mouse model, nor did they exhibit reduced colonization potential compared with wild type in competition experiments.

The hemolysin (HlyA) produced by many El Tor and non-O1 strains of *Vibrio cholerae* has been shown to be a pore-forming toxin and has been implicated as a virulence determinant (4, 25, 28, 29, 63). The production of HlyA is controlled by levels of available iron, and thus, it has been suggested that HlyA may be involved in iron scavenging (54). HlyA expression is also upregulated by the DNA-binding protein HlyU, and studies of a *hlyU* null mutant suggest that HlyU may also regulate the expression of additional virulence determinants (63).

The *hlyA* locus is closely linked to a number of other potential virulence determinants. A lecithinase-phospholipase gene, *lecA*, is transcribed divergently from *hlyA* (37), and downstream of *hlyA* lies *hlyB*, whose gene product is highly homologous to methyl-accepting chemotactic proteins (Mcps) which are involved in signalling the flagellar motor in response to environmental stimuli (44). The *hlyC* gene located downstream of *hlyB* was also identified (3) and was shown to be transcriptionally induced during infection in an infant mouse model of cholera, suggesting that it plays a role during infection of the host (13). Based on the previously noted homology of HlyC with lipases of other bacterial species, such as the *Pseudomonas aeruginosa* LipA protein (13, 14), we have renamed *hlyC* *lipA*.

The genetic organization of the *hlyA* locus suggests that it may be part of a pathogenicity island (PI). The hemolysin gene cluster of uropathogenic strains of *Escherichia coli* is located on the PI and is associated with genes involved in uropathogenicity, such as determinants encoding P-related fimbriae (10) or genes associated with pap pilus production (56). A PI has been identified for strains of *V. cholerae* O1 and O139, comprising the *tcp-acf* gene cluster, an integrase gene, and the regulatory *toxT* gene (39).

In this report, we have further analyzed the region downstream of *hlyAB* and as a result have characterized the lipase operon, *lipAB*, of *V. cholerae*; based on homology, we have also identified a metalloprotease gene, *prtV*. Assessment of both the 50% lethal doses (LD_{50}s) and colonization abilities of *lipA* and *prtV* mutants has been made with the infant mouse model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *V. cholerae* O1 strain O17 of the El Tor biotype and its *lipA* (V1218) and *prtV* (V1219) derivatives (this study) were grown in Luria broth with shaking at 37°C. *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used as a host to propagate all plasmid constructs (except pCVD442) and was grown in nutrient broth (Oxoid). Transformation of plasmid DNA into *E. coli* was performed with $CaCl_2$-treated cells as described by Brown et al. (12), while electroporation into *V. cholerae* was performed as described by Stoebner and Payne (54). To conjugate DNA from *E. coli* (S17-1λ*pir*) (19) into *V. cholerae*, overnight cultures of donor and recipient

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strains were mixed at a ratio of 1:10, centrifuged at 3,000 × *g* for 10 min (JA20 rotor; Beckman, Palo Alto, Calif.), and resuspended in 0.3 ml of Luria broth (Difco-Bacto). The mating mixture was then spread onto a 0.45-μm-pore-size Millipore filter disc laid on a Luria agar plate, and after 4 to 6 h at 37°C the bacteria were resuspended in 10 ml of saline and various 10-fold dilutions were plated on selective media. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml; and rifampin, 100 μg/ml. The plasmids used were pCVD442 (21), pBluescript SK/KS (Stratagene, La Jolla, Calif.), pGem52f(+) (Promega), and pCactus (16). The Km^r cartridge (Pharmacia LKB, Uppsala, Sweden) was isolated from pUweKT (55).

DNA methods and sequencing. Standard molecular biological techniques were performed according to Sambrook et al. (51). Restriction endonucleases were purchased from either Boehringer Mannheim or New England Biolabs and were used according to the manufacturers' instructions. Sequencing kits for dye primer sequencing were obtained from Applied Biosystems. Sequencing was carried out with a model 373A Applied Biosystems automated sequencer. The sequence was collated and analyzed with SeqEd (Applied Biosystems) and DNAsis (LKB-Hitachi). For Southern hybridization and colony immunoblotting, Hybond-N membranes (Amersham) were used essentially as recommended by the manufacturer. Detection of target DNA with digoxigenin (DIG)-labelled probes was performed with the enhanced chemiluminescence system (ECL; Amersham) as described elsewhere (61).

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed in 15% gels by a modification of the procedure of Lugtenberg et al. (42) as described previously by Achtman et al. (1). Samples were heated at 100°C for 3 min in SDS sample buffer prior to loading. The gels were then stained with 0.4% Coomassie brilliant blue R250 and destained with 5% acetic acid. Molecular mass standards (Pharmacia) are given in kilodaltons.

PCRs. PCR amplification was carried out by standard protocols with the oligonucleotides described below, allowing for 1 min per kb of PCR product. *Taq* polymerase from Hoffmann-La Roche was used in all PCRs. PCR products were ligated directly into pGem-T (Promega) and transformed into DH5α. The probe used in the isolation of pPM4355 was a 700-bp DIG-labelled PCR product generated from oligonucleotide 173 (5′ CACGGCATTCCAGAATCA 3′) and M13 reverse primer. The probe used in the isolation of pPM5105 was a DIG-labelled PCR product generated from oligonucleotides 2208 (5′ TGTTTGCAC AATATGTGC 3′) and 2209 (5′ TGGATTTGGGCACCTTCG 3′). These fragments were labelled during a PCR with DIG-11-dUTP (Boehringer Mannheim) according to the manufacturer's instructions. Oligonucleotides used in the construction of pPM4356 were 2160 (5′ TGCCTCAACCAAAGTTGG 3′) and 2158 (5′ GTCACTAAAGGCCGAGAG 3′).

T7 protein expression system. Plasmids were transformed into DH5α containing pGP1-2, which carries the T7 RNA polymerase under the control of the bacteriophage lambda P_L promoter. The method of Tabor and Richardson (57) was followed for overexpression of the cloned gene. Proteins were labelled with L-[^{35}S]methionine (Amersham) in the presence of rifampin, and after electrophoretic separation in SDS on 15% polyacrylamide gels, the proteins were detected by autoradiography of the dried gels.

Liquid hemolysis assays. Assays were performed with *V. cholerae* culture supernatants that were harvested at various stages of cell growth and were filtered through a 0.22-μm-pore-size Millipore filter. The culture supernatants were mixed with an equal volume of a 5% suspension of washed sheep erythrocytes (sRBCs). Samples were incubated at 37°C, and aliquots were taken at various time points and pelleted in a microcentrifuge for 45 s. Hemolytic activity (hemoglobin release) was assessed by measurement of change in the A_{414}. Hemolysis was expressed as a percentage of total lysis as determined by lysis of sRBCs with 2% SDS.

Lipase activity assay. Strains of *V. cholerae* were tested for lipase activity by inoculating Luria-Bertani agar supplemented with emulsified tributyrin and incubation at 37°C for 24 h. The agar plates were prepared by adding 1 g of gum arabic (Sigma Chemical Co.) and 10 ml of tributyrin (Sigma Chemical Co.) per liter of Luria-Bertani agar and emulsifying with a homogenizer prior to pouring (13).

Infant mouse studies. The in vivo consequences of introducing various mutations into the O17 strain were assessed by the infant mouse cholera model. The virulence of the parent and that of the isogenic mutant were compared by performing simultaneous (48-h) LD_{50} titrations in young Swiss mice (2.4 to 2.7 g) as described elsewhere (7). In addition, competition experiments (8) were performed to compare the colonization potentials of mutant strains with that of the wild type. These involved the administration of a mixed inoculum of the parent and mutant strains, with harvesting of intestinal contents 24 h later. Any significant change in the ratio of mutant to parent bacteria indicates a difference in colonization potential.

Primer extension analysis. Total cellular RNA was prepared by the hot phenol method as described elsewhere (62). A total of 10 to 20 μg of RNA and approximately 10 ng of oligonucleotide primer (5′ labelled with [γ-^{32}P]dATP and T4 polynucleotide kinase) were ethanol precipitated, dried, and resuspended in 12 μl of water. Extension reactions were performed with Superscript II RNase H^- Reverse Transcriptase (Gibco BRL) according to the manufacturer's instructions. After the extension reactions were performed, the samples were RNase treated, phenol extracted, and ethanol precipitated. Extension products were resolved on 6% polyacrylamide–urea gels. Sequencing reactions were performed with the same oligonucleotide primers on supercoiled plasmid DNA templates.

Nucleotide sequence accession number. The DNA sequence data from this study are available under accession no. X16945.

RESULTS

Analysis of the nucleotide sequence of the region downstream of *hlyAB*. The *V. cholerae* O1 *hlyA* and *hlyB* genes and their products have previously been characterized (2, 3, 43, 50) and are encoded on a 6.4-kb *Pst*I fragment (Fig. 1) of pPM431 (43). An additional gene, *lipA* (previously designated *hlyC*), was identified downstream of *hlyAB* (Fig. 1) and was predicted to encode a 18.3-kDa cell-associated protein with no signal peptide (3, 14). This open reading frame (ORF) was predicted to be a triacylglyceride-specific lipase based on similarity to other bacterial lipases (13, 14). Due to the close proximity of *lipA* to *hlyA*, we wanted to determine if the product of *lipA* is involved in the hemolytic activity of *V. cholerae*. To facilitate characterization of this protein, the gene was cloned behind an inducible promoter to enable overexpression. This was achieved by subcloning the terminal 1.19-kb *Hin*dIII/*Pst*I fragment of the 6.4-kb *Pst*I fragment of pPM431 into the *Hin*dIII/*Pst*I sites of the pBluescript SK polylinker to generate pPM2656 (Fig. 1). Expression of *lipA* from pPM2656 was achieved with the T7 RNA polymerase-promoter system of Tabor and Richardson (57). A 29-kDa protein produced by pPM2656 (see Fig. 5A, lane 5), which disagrees with the previously reported size of 18 kDa, was detected (3, 14). To further examine this inconsistency, *lipA* was resequenced and several differences from previous reports were detected (3, 14). A CG-to-GC transition (Arg to Ala) was found at nucleotides (nt) 5724 and 5725 of the 6.4-kb *Pst*I fragment of pPM431 (Fig. 1), and after 6,078 bp, a C leading to a shift in the reading frame was inserted. This opens the reading frame to the end of the fragment with no detectable stop codon, and thus pPM431 does not contain the complete *lipA* gene.

To obtain the complete *lipA* gene, it was necessary to map restriction sites downstream of the 6.4-kb *Pst*I site on the *V. cholerae* O1 chromosome by Southern blot analysis. *Eco*RV and *Eco*RI restriction sites were mapped 3 and 4.7 kb downstream of the 6.4-kb *Pst*I site, respectively (Fig. 1). Two subgenomic chromosomal libraries of *V. cholerae* were generated with *Eco*RV fragments of 5 to 6 kb or *Eco*RI/*Bam*HI fragments of approximately 6 to 7 kb. The libraries were screened with DIG-labelled PCR probes in a DNA colony immunoblot. Two constructs were isolated, pPM4355 and pPM5105, containing a 5.6-kb *Eco*RV fragment and a 6.4-kb *Eco*RI/*Bam*HI fragment, respectively (Fig. 1). Sequencing 3′ to the 6.4-kb *Pst*I site revealed a continuation of the ORF for *lipA* to 933 bp, which is now predicted to encode a 33-kDa protein (Fig. 1).

It was also reported that translation of *lipA* initiates from the AUG codon at bp 5578 to 5580 of the *Pst*I fragment and that LipA does not possess an amino-terminal signal sequence (3, 14). However, as the members of this family of triacylglyceride-specific lipases are secreted proteins, we predict that the codon UUG (leucine), which lies 27 amino acids downstream of the previously reported start site, is the translational initiation site for *lipA*. Initiation of translation from UUG would generate an excellent amino-terminal signal sequence according to the constraints of von Heijne (60). While AUG is used as the preferred initiation codon in prokaryotes (91%) (24), this does not necessarily imply that *lipA* will be poorly translated, since other factors such as a good ribosome-binding site (RBS) (AGGA) with optimal spacing (7 bases from the initiation codon of *lipA*) may compensate for the nonoptimal initiation codon (40).

Beyond the termination codon of *lipA* are two additional

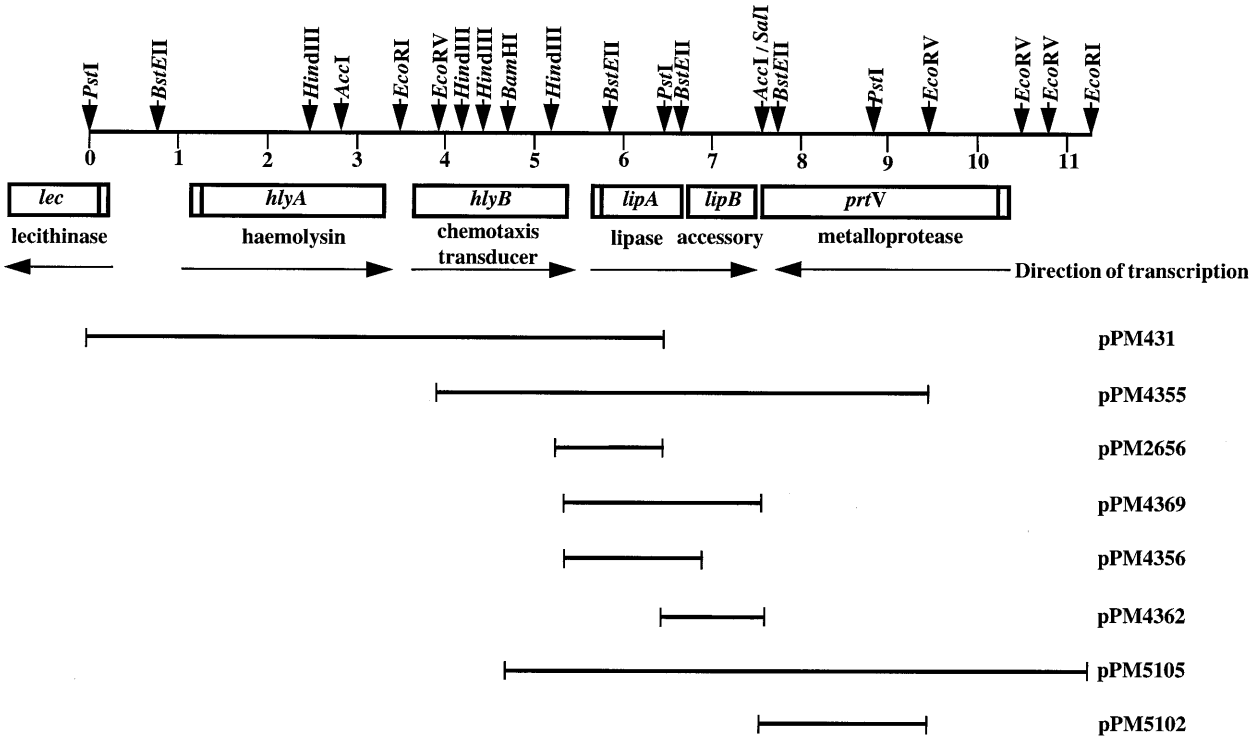

FIG. 1. Genetic organization of the *hly* and *lip* loci and the *prtV* gene of *V. cholerae* O1. Genes of the *hly* locus are labelled *hlyA* and *hlyB*, and genes of the *lip* locus are labelled *lipA* (formerly *hlyC*) and *lipB*. The metalloprotease gene *prtV* is transcribed in the opposite direction. The upper line shows relevant restriction enzyme sites of this region. Plasmid pPM431 is shown and has been described elsewhere (43). Plasmids pPM4355, pPM2656, pPM4369, pPM4356, pPM4362, pPM5105, and pPM5102, which were used in this study, are shown. The orientations of inserts are such that the T7 promoter is to the left of each insert (except for pPM5105, in which the T7 promoter is to the right of the insert).

ORFs, *orf1* and *orf2*, with predicted molecular masses of 32.6 and 102 kDa, respectively. *orf1* lies 9 bp downstream of the stop codon of the preceding *lipA* and its potential RBS, AG GAG, overlaps the stop codon of *lipA*, suggesting that they are translationally coupled. *orf2* lies downstream of *lipA* and *orf1* but is transcribed in the opposite direction and is preceded by two copies of a putative RBS, which are present on a 10-bp tandem repeat sequence, TAAGGAAATA (the RBS is underlined). Multiple RBSs have previously been reported for the chloramphenicol acetyltransferase gene, which is preceded by three functional RBSs (5). A stem-loop structure resembling a *rho*-independent terminator was found in the intergenic region between *orf1* and *orf2* and probably functions as a bidirectional terminator (Fig. 2). The free energy of the structure (ΔG, -22.4 kcal mol^{-1}) indicates that it would be highly favorable (59), and multiple A residues are present 5′ and 3′ to the stem loop.

***lipA* and *orf1* are highly homologous to the lipase and accessory lipase genes of *Pseudomonas* spp.** A search of the database with the 33-kDa LipA of *V. cholerae* shows strong homology with lipase genes of *Pseudomonas* spp. (Table 1). The highest degree of similarity was observed with *P. aerugi-*

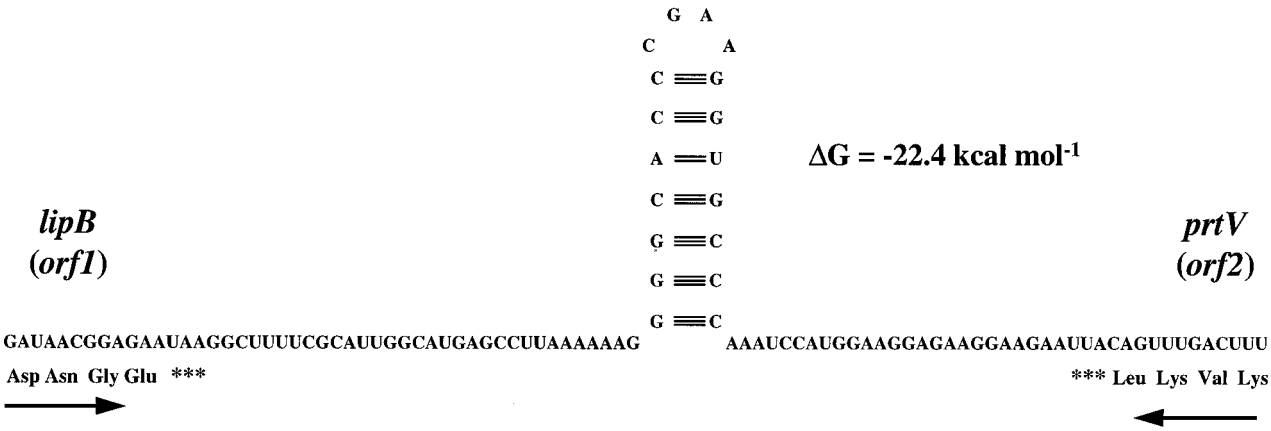

FIG. 2. The stem-loop structure ($\Delta G = -22.4$ kcal mol^{-1}) in the intergenic region of *lipB* and *prtV* is proposed to be a bidirectional terminator. Arrows indicate the directions of transcription.

TABLE 1. Comparison of *V. cholerae* LipA with other lipases

LipA source	% Identity[a]					
	V. cholerae[b]	*P. aeruginosa*[c]	*P. fragi*[d]	*A. calcoaceticus*[e]	*P. glumae*[f]	*P. cepacia*[g]
V. cholerae	100 (312)	59.1 (308)	47.3 (262)	45.2 (301)	42.8 (228)	42.6 (216)
P. aeruginosa		100 (311)	45 (262)	51.6 (275)	39.2 (237)	37.7 (231)
P. fragi			100 (277)	39 (272)	42.9 (182)	43.4 (182)
A. calcoaceticus				100 (323)	36.2 (240)	37.5 (216)
P. glumae					100 (358)	78 (364)
P. cepacia						100 (364)

[a] Determined with FASTA as implemented by PROSIS version 7 (LKB-Hitachi software). Numbers in parentheses are numbers of amino acids over which the indicated identity occurs.
[b] Accession no. X16945.
[c] Accession no. JT0958.
[d] Accession no. P08658.
[e] Accession no. X80800.
[f] Accession no. Q05489.
[g] Accession no. A16316.

nosa (59.1%). Homology included the signal peptide sequence which supports our prediction for an alternative start site (UUG) for LipA.

Sequence alignment studies reveal that lipases and lipoprotein lipases have a consensus pentapeptide Gly-x1-Ser-x2-Gly (where x1 is His in prokaryotic lipases), similar to the one common to serine proteases (6, 15, 20). Crystallization and three-dimensional (3D) structure analysis of eukaryotic lipases have revealed a catalytic triad of residues Ser-Asp-His (11, 64), which is also conserved among *Pseudomonas* lipases (15, 32). The serine residue is common to both the pentapeptide and the catalytic triad. The sequence alignment of *V. cholerae* LipA with the lipase of *P. aeruginosa* showed that both the pentapeptide Gly-His-Ser-His-Gly (108 to 112) and catalytic triad Ser110 Asp256 His278 regions are conserved (Fig. 3).

The *P. aeruginosa* lipase has a single functional disulfide bond connecting residues Cys183 and Cys235 (32), and the presence and the position of the Cys residues are conserved in *Pseudomonas cepacia* (35) and *Pseudomonas glumae* (22), as well as those of the *V. cholerae* lipase shown here, with Cys212 and Cys262 highlighted in Fig. 3.

The amino acid sequence of *orf1* showed 29 and 25% identity to accessory lipase proteins LipB of *Acinetobacter calcoaceticus* (38) and LipH of *P. aeruginosa* (65), respectively. Based on this homology, *orf1* was designated *lipB*. Most lipases from *Pseudomonas* spp. require a downstream accessory gene for activity, as reported for *P. aeruginosa* PAO1 and TE3285, *P. cepacia* DSM3959, *P. glumae*, and *Pseudomonas* sp. strain 109 (15, 23, 30, 35, 65). However, this accessory protein was not detected in all *Pseudomonas* strains examined (33, 58).

A hydropathy plot of the derived amino acid sequence of *V. cholerae* LipB reveals a distinct hydrophobic region at the N

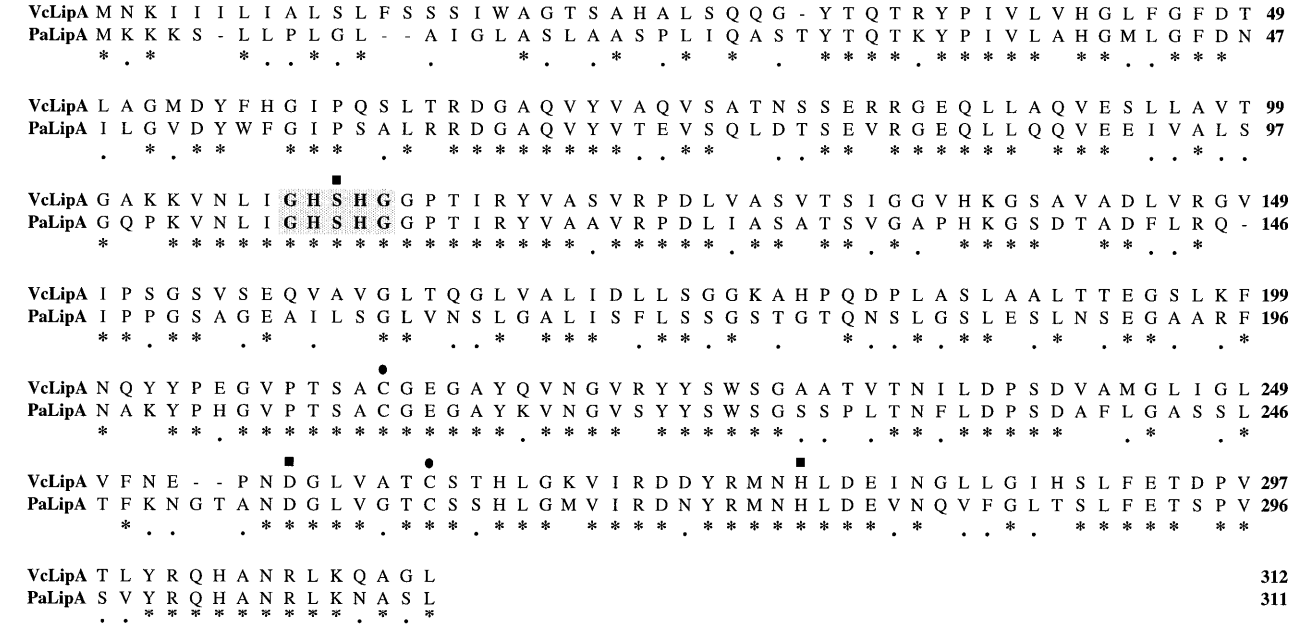

FIG. 3. Comparison of *V. cholerae* O1 LipA (VcLipA) with LipA from *P. aeruginosa* (PaLipA). The pentapeptide consensus motif (G-x1-S-x2-G) found in the active site of this family of lipases is highlighted. Squares indicate the conserved residues (Ser, Asp, and His) of the catalytic triad. The cysteine residues conserved among *Pseudomonas* spp. and present in *V. cholerae* LipA are indicated by closed circles. The homologous proteins were initially found with BLAST e-mail searches of the databases at NCBI. The alignments of the amino acid sequences were performed with Clustal V (26, 27). Asterisks, identical residues; dots, conserved residues with similar properties. Gaps (dashes) have been introduced to optimize alignment.

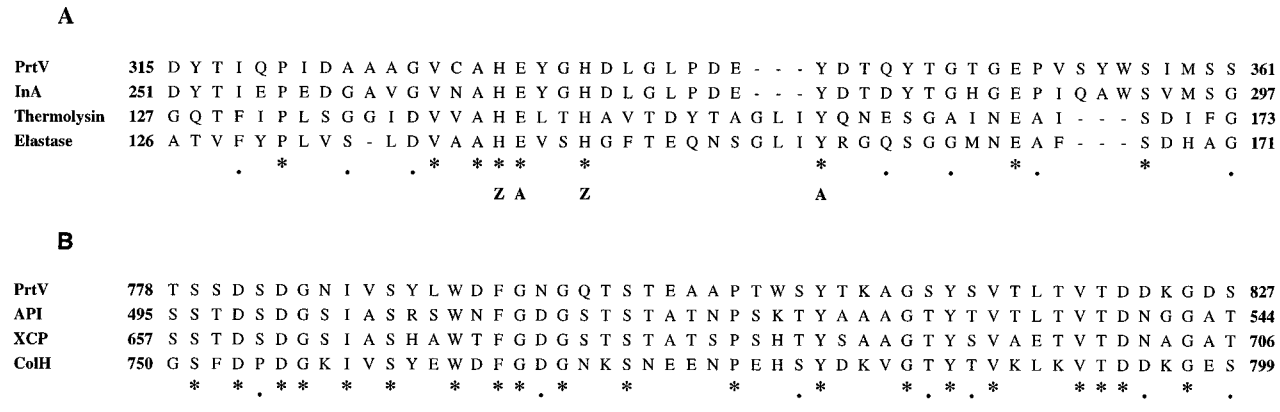

FIG. 4. Conserved domains in PrtV. (A) A comparison of the region around the known active sites and zinc-binding domains (indicated by A and Z, respectively) of *Bacillus thermoproteolyticus* thermolysin with those predicted for *B. thuringiensis* InA, *P. aeruginosa* elastase, and *V. cholerae* PrtV. (B) Comparison of PrtV with *Achromobacter* protease I (API), *Xanthomonas* carboxyl proteinase (XCP), and *C. histolyticum* collagenase (ColH). The function of this highly conserved region is currently unknown, and it is the only region of homology among these proteins. This region is not conserved in *B. thuringiensis* InA. The homologous proteins were initially found by BLAST e-mail searches of the databases at NCBI. Multiple alignments of the amino acid sequences were performed with Clustal V (26, 27). Asterisks, identical residues, dots, conserved residues with similar properties. Gaps (dashes) have been introduced to optimize alignment. The numbers of the first and last amino acid are indicated for each protein.

terminus between residues 4 and 28 (data not shown). This may be indicative of a membrane-associated domain, since the hydrophobic region would be able to span the membrane bilayer. Indeed, Frenken et al. (23) showed that LipB of *P. glumae* is anchored in the inner membrane by a hydrophobic transmembrane helix domain located at the N terminus, while the rest of the protein is periplasmically located.

***orf2* is homologous to a metalloprotease produced by *Bacillus thuringiensis*.** The amino acid sequence of *orf2* located downstream of *lipAB* was found to be highly homologous to that of the secreted neutral metalloprotease, immune inhibitor A (InA), of *B. thuringiensis* var. *alesti*, and consequently *orf2* was named *prtV* (protease of *V. cholerae*). The InA protease has been shown to specifically cleave antibacterial proteins produced by the insect host (17) and has been implicated as a virulence determinant of *B. thuringiensis* (41). The primary sequence of InA has the zinc-binding and catalytic active site residues present in various other metalloproteases, including thermolysin (41), and the significant residues are highlighted in Fig. 4A. The zinc-binding signature pattern present in PrtV is sufficient to detect members of the zinc-metallopeptidase superfamily of proteins (34, 46).

PrtV also has homology with a different group of proteases, including *Achromobacter* protease I, *Xanthomonas* carboxyl proteinase, and *Clostridium histolyticum* collagenase (accession nos. sp15636, D83740, and D29981, respectively). While the overall homology among these proteins is limited, there is a small highly conserved region present (Fig. 4B), the function of which is currently unknown. This region was not conserved with *B. thuringiensis* InA.

Expression of LipA and PrtV. The *lipA*, *lipB*, and *prtV* genes of *V. cholerae* potentially encode 33-, 32.6-, and 102-kDa proteins, respectively. To confirm this, plasmids with various combinations of the genes were constructed for expression studies. pPM4356 encodes only LipA and was constructed by PCR amplification of the region between nt 5426 and 6704 (Fig. 1), with pPM4355 as a template. The derived 1.3-kb fragment was ligated into pGem-T. A 2.5-kb *Pst*I fragment of pPM4355 (sites lie from nt 6446 to 8984 of Fig. 1) was isolated and cloned into pBluescript KS. This intermediate construct was then digested with *Acc*I (sites lie at nt 7686 [Fig. 1] and in the vector polylinker), and the intervening 1.3-kb fragment was deleted. The resulting construct was designated pPM4362 (Fig. 1) and encoded only LipB. Construct pPM4369, carrying both *lipA* and *lipB*, was generated by combining the inserts of pPM4356 (*lipA*) and pPM4362 (*lipB*). An *Apa*I/*Pst*I digest of pPM4356 was used to liberate the 1.3-kb insert carrying *lipA*. The *Apa*I site lies in the polylinker, and the *Pst*I site lies at nt 6446 of the fragment shown in Fig. 1. This 1.3-kb *Apa*I/*Pst*I fragment was ligated together with the 1.2-kb *Pst*I/*Acc*I fragment isolated from pPM4362 with the common *Pst*I site, and this fragment was ligated into the *Apa*I/*Acc*I sites of pBluescript SK. The clones pPM4355, pPM4369, pPM4356, and pPM4362 (Fig. 1) were orientated such that *lipA* and *lipB* were under the control of the bacteriophage T7 promoter.

Protein expression was achieved with the T7 promoter-RNA polymerase system of Tabor and Richardson (57). Two proteins migrating at 33 and 30 kDa were produced by strains carrying pPM4356, pPM4369, and pPM4355 (Fig. 5, lanes 1, 3, and 4, respectively), all of which encode *lipA*. Since pPM4356 encodes only *lipA* and LipA has predicted molecular masses of 33 kDa prior to and 30 kDa after the removal of the signal sequence, we conclude that the two observed bands (A and B in Fig. 5) represent unprocessed and processed LipA. Truncated LipA of 29 kDa was produced by pPM2656 and has been discussed earlier (Fig. 5, lane 5).

We were unable to detect a band with the predicted molecular mass of LipB (32.6 kDa) (Fig. 5, lane 2). LipB has only one methionine residue, the initiation residue, which limits labelling with [^{35}S]methionine and hence detection by autoradiography. Furthermore, the initiation methionine may be processed as observed with other proteins (9). pPM5105 (Fig. 1) produced a protein of approximately 102 kDa (Fig. 5B), which is consistent with the coding capacity of the *prtV* gene.

Primer extension analysis of *lipA* and *prtV*. Primer extension analysis was performed to map the 5′ end of the *lipA* and *prtV* mRNAs and to localize potential promoter regions for these genes. Total RNA from *V. cholerae* O17 was hybridized to *lipA* oligonucleotide 2205 (5′ AGACAATTGGATAACGG 3′) or *prtV* oligonucleotide 2315 (5′ GAGCGTATAATCCACTGC 3′), and extension reactions were performed. Two major extension products were obtained for *lipA* (data not shown), and the 5′ ends mapped approximately 78 and 117 nt upstream of the *lipA* TTG codon. Several other higher-molecular-weight

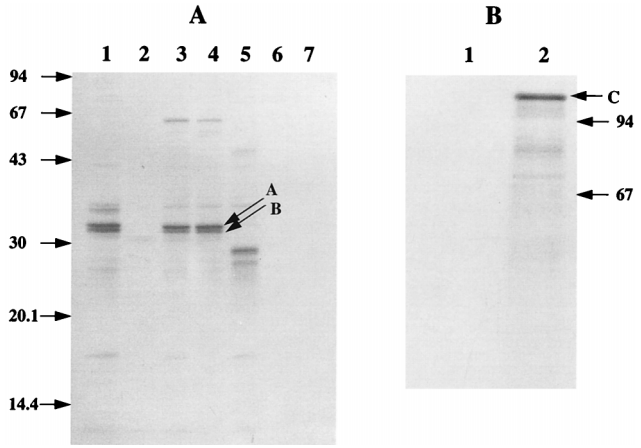

FIG. 5. Synthesis of LipA and PrtV with the T7 RNA polymerase-promoter system of Tabor and Richardson (57). Proteins were labelled with [^{35}S]methionine, and whole-cell fractions were subjected to SDS-15% PAGE followed by autoradiography of the dried gel. The plasmids used are described in the legend to Fig. 1. The molecular mass standards are shown in kilodaltons. (A) Lanes 1 to 7, pPM4356, pPM4362, pPM4369, pPM4355, pPM2656, pBluescript SK, and pGem52f, respectively. Unprocessed and processed LipA are indicated by arrows A and B, respectively. LipB was not detected and is discussed further in the text. (B) Lanes 1 and 2, pGem3Zf and pPM5105, respectively. PrtV is indicated by arrow C.

extension products were observed but could not be accurately mapped, and the presence of these products may indicate that processing of a polycistronic mRNA occurs. Potential σ^{70} consensus promoter sequences at appropriate spacing from the 5′ end were identified (Fig. 6). Two extension products were obtained for *prtV*. The 5′ ends mapped 39 nt (minor product) and 300 nt (major product) upstream of the *prtV* ATG codon (Fig. 6). A potential −10 consensus sequence precedes the 5′ end of the minor product, and potential −10 and −35 sequences precede the 5′ end of the major product (Fig. 6).

Construction of a *lipA* chromosomal mutant in *V. cholerae* O17. To investigate the role of *lipA*, a specific mutation was constructed in *V. cholerae* O17 by using the suicide vector pCactus to introduce a Km^r cartridge into the gene. pCactus has a temperature-sensitive replicon and encodes Cm^r. The replicon is inoperative at 42°C but supports plasmid replication at 30°C. It also encodes the *sacB* gene from *B. subtilis*, the product of which is toxic to bacteria when they are grown in the presence of sucrose (36).

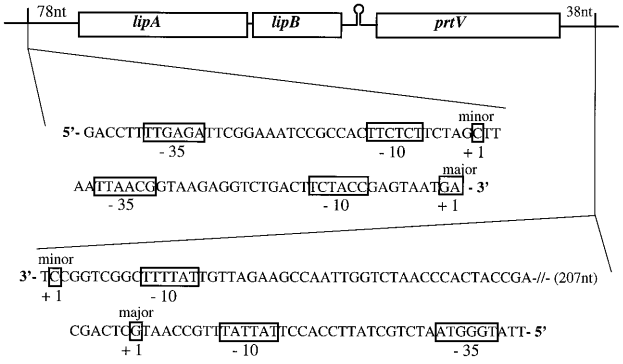

FIG. 6. DNA sequences of the predicted promoter region of *lipA* and the divergently transcribed *prtV*. Potential −10 and −35 promoter sequences are indicated. The transcriptional start sites determined by primer extension are indicated by +1.

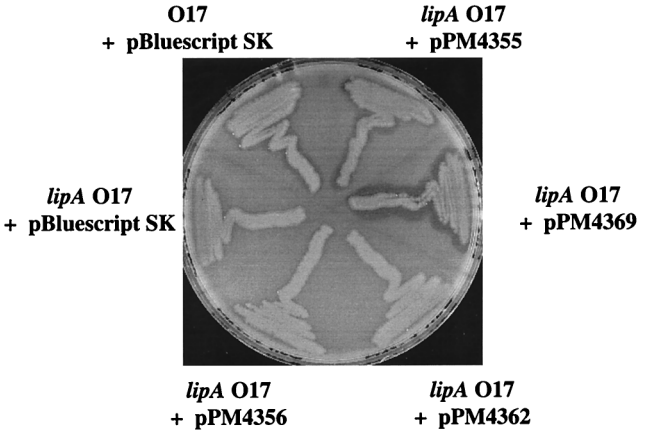

FIG. 7. A comparison of the lipase activities of *V. cholerae* O1 and its isogenic *lipA* mutant (V1218) containing various plasmids (Fig. 1), as indicated by a zone of clearing on Luria-Bertani agar containing emulsified tributyrin after an incubation period of 24 h.

A 1.19-kb *Sal*I/*Bam*HI fragment carrying most of *lipA* was isolated from pPM2656 and ligated into the *Sal*I/*Bam*HI sites of pCactus. The DNA was transformed into DH5α and grown at 30°C in the presence of chloramphenicol, and the resulting construct was designated pPM2657. A 1.2-kb *Hin*cII Km^r cartridge was then inserted into the end-filled *Bst*EII restriction site of pPM2657, 981 bp downstream of the *lipA* start codon. pCactus carrying *lipA*::Km^r was isolated from *E. coli* after growth at 30°C and was designated pPM4357.

pPM4357 was electroporated into *V. cholerae* O17 and grown at 30°C in the presence of kanamycin and chloramphenicol. Aliquots of an overnight culture of this strain were plated on kanamycin-containing brain heart infusion medium and grown overnight at 42°C (growth of the strain at the nonpermissive temperature of 42°C in the presence of kanamycin selects for strains in which recombination with host DNA has occurred, since the plasmid pCactus cannot replicate at this temperature). The resulting colonies were then inoculated into Luria broth in the presence of kanamycin and grown overnight at 30°C to allow for the resolution of cointegrates of plasmid and host chromosomal DNAs. Cells which did not resolve the cointegrate were selected against by growth in the presence of sucrose at 30°C. Selection for Km^r Cm^s sucrose^r colonies at 37°C ensured that a double-crossover recombination event had occurred in *V. cholerae*, such that the effective copy of *lipA* had been replaced with the inactive copy, and the plasmid pCactus was no longer present. The putative *lipA*::Km^r chromosomal mutant V1218 was confirmed by both PCR and Southern hybridization analysis (data not shown).

LipA and LipB are both essential for lipase activity. Since *lipA* and *lipB* are predicted to encode a lipase and accessory lipase based on sequence homology, we examined the effect of the *lipA* mutation on lipase activity. Agar plates containing emulsified tributyrin were inoculated with various strains and incubated at 37°C for 24 h. While the parental O17 strain displayed a zone of clearing consistent with lipase activity, the isogenic *lipA* mutant V1218 failed to do so (Fig. 7), in agreement with the previous findings of Camilli and Mekalanos (13). In order to complement the LipA defect and define the region required to do so, a variety of plasmid constructs were electroporated into V1218. Plasmid pPM4355 containing the 5.6-kb *Eco*RV fragment carrying *lipA*, *lipB*, and additional downstream DNA was able to restore the lipase activity of

V1218, confirming the isolation of a lipase operon of *V. cholerae*. Plasmid pPM4369 carrying only *lipA* and *lipB* was similarly able to restore activity. However, the independent expression of either *lipA* or *lipB* did not restore lipase activity to V1218 (Fig. 7). Therefore, we conclude that *V. cholerae* requires the product of both the *lipA* and the *lipB* genes for lipase activity.

Camilli and Mekalanos (13) have suggested that *lipA* may be coregulated by HlyU. HlyU is known to upregulate *hlyA* expression, and readthrough of the intergenic terminator may also affect expression of the downstream gene, *hlyB* (62). To determine whether *lipAB* expression is regulated by HlyU, strains O17, O17 *hlyU* (V876) (63), and O17 carrying the *hlyU* clone pPM3039 (62) were compared for lipase activities on emulsified tributyrin agar. There was no detectable difference in the zones of clearing (data not shown). In addition, initial investigations of these strains show no difference in *lipA*-specific mRNA levels, as determined by primer extension analysis (data not shown). However, we cannot rule out the possibility that HlyU is involved in *lipA* expression in vivo.

Mutating the *prtV* gene of *V. cholerae*. To elucidate a role for the putative metalloprotease PrtV in *V. cholerae*, a chromosomal mutation was constructed by inserting a Km^r cartridge into the *prtV* gene. A Km^r cartridge residing on a 1.2-kb *Pst*I fragment was inserted into the *Pst*I site of pPM5102 (Fig. 1). The entire insert containing the Km^r cartridge was subsequently recloned into the suicide vector pCVD442 (21), with the flanking *Sal*I and *Sph*I restriction sites, to generate pPM5104 (Fig. 1). pCVD442 is Ap^r and also encodes SacB. Plasmid pPM5104 was transformed into *E. coli* S17-1λ*pir* to enable replication from the R6K *ori* in pCVD442, and this strain was used to conjugate the plasmid into *V. cholerae* O17. A Km^r O17 exconjugant was isolated and plated onto Luria broth (ampicillin, 6% sucrose, lacking NaCl) at 30°C, and a Km^r Ap^s colony was isolated and confirmed to be a *prtV* mutant of *V. cholerae* by PCR and Southern hybridization analysis (data not shown).

Effects of the *lipA* and *prtV* mutations on hemolytic activity. The *lipA* (V1218) and *prtV* (V1219) mutants of *V. cholerae* O17 were assayed for hemolytic activities to determine the effects of these mutations. Culture supernatants of the parental strain O17 and mutated strains V1218 and V1219 were collected at various stages of cell growth, filtered, and tested for hemolytic activity in a liquid hemolysis assay. The hemolytic activity of the O17 strain was unaffected by a mutation in either the *lipA* or *prtV* gene (data not shown).

Effects of the *lipA* and *prtV* mutations on virulence. The in vivo consequences of introducing various mutations into the O17 strain were assessed by the infant mouse cholera model. The degrees of virulence of the parent and of isogenic mutants were compared by performing simultaneous (48-h) LD_{50} titrations. The values obtained show that neither V1218 (LD_{50} of 5.6×10^3, compared with 4.4×10^4 for O17) nor V1219 (LD_{50} of 8.8×10^3, compared with 9.2×10^3 for O17) was attenuated.

In addition, competition experiments were performed to compare the colonization potentials of the mutant strains with that of wild-type O17. For both mutants, the output ratios (ORs) of mutant to parent bacteria recovered at 24 h were not significantly different from the ratio present in the challenge inoculum. For V1218, the mutant-to-parent input ratio was 2.3, and the median OR was 1.2 ($n = 7$); for V1219, the input ratio was 1.0, and the median OR was 2.6 ($n = 6$).

DISCUSSION

Linked to the *hly* locus of *V. cholerae* are a number of potential virulence-associated determinants, including a hemolysin, lecithinase, and methyl-accepting chemotactic protein. Sequence analysis of the region downstream has revealed three additional ORFs, and the corresponding genes were named *lipA*, *lipB*, and *prtV* (the genetic organization of the extended locus is summarized in Fig. 1). This lipase operon of *V. cholerae* consists of *lipA* and *lipB*, which we predict are translationally coupled, since the stop codon for *lipA* overlaps the RBS for *lipB*. Downstream of *lipAB* lies *prtV*, which is transcribed in the opposite direction and which, based on sequence homology, is predicted to be a metalloprotease. A potential rho-independent terminator is present in the intergenic region between *lipAB* and *prtV* and may therefore function in bidirectional termination of transcription. Primer extension analysis has located potential start sites for both *lipA* and *prtV* transcription, and potential σ^{70} consensus promoter sequences were identified.

The *lipA* gene was previously known as *hlyC* and was reported to encode an 18.3-kDa protein with no potential signal sequence (3, 14, 44). However, by resequencing this gene and overexpressing its protein product, we have established that it actually encodes a 33-kDa protein, with a potential signal peptide at the amino terminus. Both unprocessed (33-kDa) and processed (30-kDa) forms of the LipA protein were detected on a polyacrylamide gel. This ORF has an atypical start codon of UUG but a good match to the consensus RBS and is translated sufficiently to be detected in T7 overexpression analysis.

The 33-kDa LipA protein of *V. cholerae* described here is highly homologous to the triacylglyceride-specific lipase of the *Pseudomonas* spp. *P. aeruginosa* (59%), *P. fragi* (47%), *P. glumae* (43%), and *P. cepacia* (43%) and *A. calcoaceticus* (45%), all of which share sequence homologies in significant regions. Almost all known amino acid sequences of neutral lipases contain the consensus pentapeptide Gly-x1-Ser-x2-Gly, where x1 is His in prokaryotic lipases (6, 15, 20), and this pentapeptide is present in LipA of *V. cholerae*. The serine residue of the pentapeptide is also conserved among serine proteases. Evidence for the presence of a serine residue at the active site was obtained by using the serine-specific inhibitor DNPP (diethyl-*p*-nitrophenyl phosphate), which completely inhibited the enzymatic activities of lipases isolated from porcine pancreas (45) and *P. aeruginosa* (32). In addition, site-directed mutagenesis of the serine residue to glycine in rat hepatic lipase resulted in the complete loss of activity (18).

V. cholerae LipA also contains the three highly conserved amino acids (Ser-Asp-His) known as the catalytic triad. This 3D triad is a well-known structural feature of the serine proteases and has been observed directly in the catalytic sites of eukaryotic lipases of *Rhizomucor miehei* (11) and human pancreas (64). The serine residue of the triad is the same serine as that of the conserved pentapeptide. The catalytic triad is conserved among lipases of *Pseudomonas* species, as demonstrated by the amino acid sequence alignment of various *Pseudomonas* lipases to the crystal structure of human pancreatic lipase (15) and modelling of the 3D structure of the *P. aeruginosa* lipases (15, 23, 30).

Immediately downstream of the *V. cholerae lipA* lies *lipB*, and expression studies of LipA, LipB, and LipAB in a *lipA V. cholerae* strain indicate that LipB is essential for lipase activity. Accessory genes with homology to *lipB* have been shown to be required for the production of active lipases, especially within the *Pseudomonas* spp. (15, 35, 65). Frenken et al. (23) have demonstrated that the accessory protein LipB of *P. glumae* is a

lipase-specific foldase and have also shown that correct folding of the lipase was essential for both enzymatic activity and translocation of the lipase across the outer membrane. LipA of *P. aeruginosa* TE3285 requires LipB for activation in a noncatalytic mode; however, there is no sequence similarity between the LipB protein and any known chaperone, suggesting that the mechanism of activation by LipB is different from that of chaperones (31, 48).

The regulation of the *lipAB* operon remains unknown. Previously, *lipA* was selected to be an in vivo-activated promoter, induced only during an infection of the host animal (13). However, we were able to detect both lipase activity and specific mRNA in vitro. *lipA* expression is not regulated (in vitro) by iron levels, by microaerophilic conditions, or by conditions that affect the expression of ToxR-regulated genes (13). The ToxRST system regulates several significant virulence determinants of *V. cholerae*, including the cholera enterotoxin and toxin coregulated pilus (49). Camilli and Mekalanos (13) have raised the possibility that *lipA* is regulated by HlyU; however, we have found no evidence to support this. The *lipAB* operon may, therefore, be regulated by a novel system in *V. cholerae*, and this aspect is worthy of further investigation.

The *prtV* gene lies downstream of the *lipAB* operon and is predicted to encode a metalloprotease, based on homology with the metalloprotease InA of *B. thuringiensis*. Functionally important zinc-binding and catalytic active site residues conserved among metalloproteases (34, 46) are also present in the amino acid sequence of PrtV. *B. thuringiensis* is an insect pathogen, and while the main insecticidal activity is due to the δ-endotoxin, InA also contributes to pathogenesis of the bacteria (41, 52, 53). However, it is interesting to note that although InA degrades the antibacterial proteins cecropin and attacin produced by the insect host, these agents show only a low level of activity in vitro against *B. thuringiensis* (17) and may therefore actually be targeted against a different protein (41).

The hemolysin (HlyA) requires proteolytic cleavage for activation (66), and the possible involvement of PrtV in this process was investigated. *prtV* null mutants were fully hemolytic in a liquid hemolysis assay; however, a variety of other proteases are also capable of processing HlyA (47); thus, a conclusion cannot be made at this stage.

Although the LipA and PrtV proteins could potentially damage host cells, mutations in either gene did not affect the colonization potential or virulence in the infant mouse cholera model. It is perhaps not surprising that no differences in in vivo behavior were observed when mutants were separately competed against the parent strain in mixed infection experiments. The products of both genes are likely to be secreted, in which case proteins produced by the wild-type vibrios might be expected to compensate the mutant strains in the provision of lipase or protease function. Since the mutants retain full virulence, neither LipA nor PrtV provides an indispensable virulence function in the O17 strain.

It is possible that the proteins are involved in the acquisition of nutrients, which appears to be a general theme of the genes encoded within this region of the *V. cholerae* chromosome (Fig. 1). The HlyA toxin is capable of host tissue damage, which would release a variety of cellular components including iron, membrane lipids, and proteins, and enzymes capable of degrading these substances (Lec, LipA, and PrtV) are encoded in the vicinity of *hlyA*. The level of HlyA production is responsive to the levels of available iron (54), an essential requirement for bacterial survival. A chemotactic transducer, HlyB, also encoded in this locus, could monitor the nutrient status of the surrounding environment and signal the bacteria to move accordingly.

In this report, we have characterized the triacylglyceride-specific lipase operon of *V. cholerae*, which encodes a 33-kDa lipase (LipA) and a 32-kDa accessory protein (LipB), both of which were essential for lipase activity. In addition, a putative metalloprotease gene, *prtV*, which specifies a 102-kDa product with homology to the InA protein of *B. thuringiensis*, was identified. This region may be part of a PI which is capable of host cell damage and/or of altering the conditions of the gut to favor growth.

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