

NOTES

Evidence that AphB, Essential for the Virulence of *Vibrio vulnificus*, Is a Global Regulator^{∇†}

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The *Vibrio vulnificus* aphB mutant was significantly less virulent than the wild type and was impaired in motility and adherence to host cells. Microarray analysis revealed that AphB of *V. vulnificus* (AphB_{Vv}) influences the expression of over 10% of the *V. vulnificus* genome. The combined results indicated that AphB_{Vv} is a global regulator contributing to the pathogenesis of *V. vulnificus*.

Microbial pathogenicity is a complex phenomenon that involves the products of many genes, called virulence factors, contributing not only to diseases but also to survival and multiplication on or within the host (20). Most of these virulence factors act cooperatively to obtain maximum effectiveness during pathogenesis, while their expression is coordinately controlled by a common global regulatory system in response to environmental signals (21). This coordinate regulation by a global regulator may facilitate the cooperation of the virulence factors and be crucial for the overall success of the infectious microorganisms during pathogenesis (21). Recently, many regulatory proteins have been implicated as important global regulators controlling the expression of numerous virulence factors in bacterial pathogens (5, 9, 19, 22).

Vibrio cholerae AphB (AphB_{Vc}) is a member of the LysR family of transcriptional regulators and plays a central role in the activation of the ToxR virulence cascade (11). AphB_{Vc} initiates the expression of the ToxR virulence cascade by directly binding to and activating the promoter of *tcpPH* in response to yet-unknown environmental signals (11, 12, 13, 14). TcpPH and ToxR function cooperatively in the regulatory cascade, leading to the biogenesis of toxin-coregulated pilus (TCP) and the production of cholera toxin (CT) (4, 7, 11). The TCP is crucial for colonizing the upper intestine (8), and the CT is responsible for the severe diarrhea caused by *V. cholerae* (10), suggesting that AphB_{Vc} is an essential transcriptional regulator modulating the virulence of the organism.

Previously, a homologue of AphB_{Vc} from *V. vulnificus*, a causative agent of food-borne diseases such as gastroenteritis and life-threatening septicemia, was identified (28). The deduced amino acid sequence of AphB from *V. vulnificus* (AphB_{Vv}) was 80% identical to that of AphB_{Vc}. However, a search of the

genome sequences of both *V. vulnificus* CMCP6 and YJ016 to find any *tcpPH* gene homologues with a substantial level of identity was not successful (S. H. Choi, unpublished data). Instead, AphB_{Vv} directly activates the transcription of *cadC*, the product of which facilitates the survival of the pathogen under acid stress (28, 29, 30). This finding indicated that the set of genes regulated by AphB may be broad rather than specific and prompted us to further characterize the functions of the regulatory protein, thus identifying its target genes on a global scale. Accordingly, the present study assessed the functions of AphB by comparing phenotypes of the *V. vulnificus* aphB mutant with those of the parental wild type in vitro and in mice. Transcriptome analysis using a *V. vulnificus* whole-genome microarray was also performed and resulted in the identification of at most 489 genes as members of the AphB regulon. Fourteen genes, randomly chosen from the pool of the newly identified genes, were experimentally verified to be regulated by AphB.

Unless noted otherwise, *V. vulnificus* strains were grown in Luria-Bertani medium supplemented with 2.0% (wt/vol) NaCl at 30°C. For transcriptome analysis, the *V. vulnificus* whole-genome TwinChip, manufactured and kindly provided by the 21C Frontier Microbial Genomics and Applications Center (Daejeon, South Korea), was used. Total cellular RNA from the *V. vulnificus* strains grown to an optical density at 600 nm of 0.5 was isolated with an RNeasy midi kit (Qiagen, Valencia, CA), and aminoallyl cDNA was synthesized using an aminoallyl cDNA-labeling kit according to the protocols of the manufacturer (Ambion, Austin, TX). The aminoallyl cDNA from the aphB mutant and that from the wild type were labeled with Cy3 and Cy5 (Ambion), respectively, and equal amounts of the labeled cDNA were combined and used to hybridize the microarray slides at 42°C for 16 h. After hybridization, the arrays were washed, dried, and scanned using GenePix 4000B (Axon Instruments, Foster City, CA). Data from three independent experiments were normalized and then analyzed using the GenePix Pro 3.0 software (Axon Instruments). The open reading frame spots that showed a 2.828-fold or greater difference

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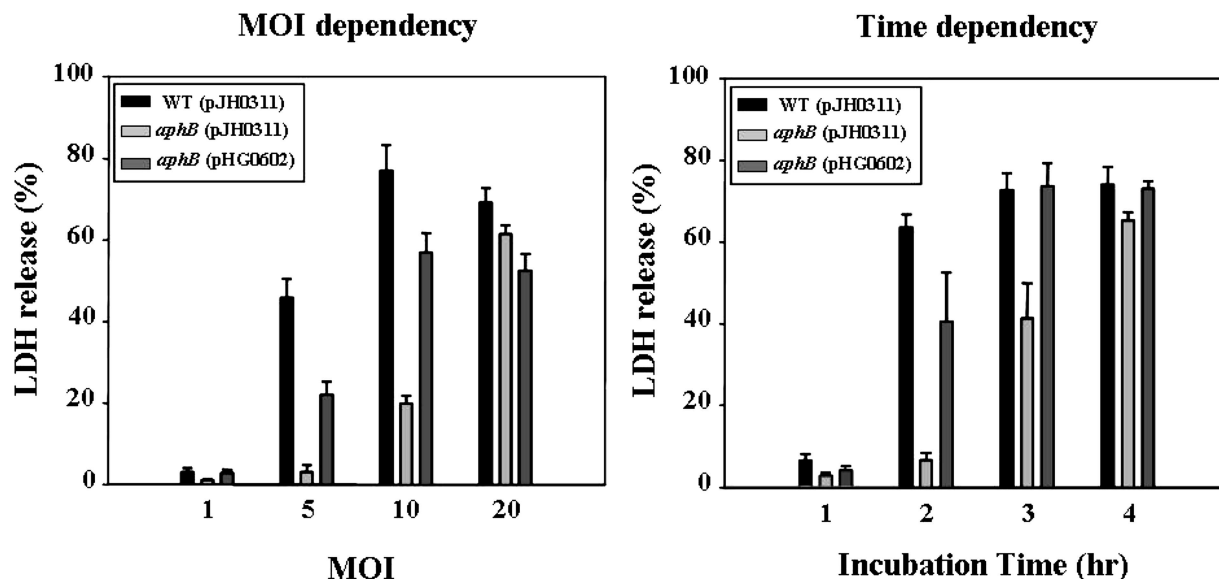


FIG. 1. Effect of *aphB* mutation on virulence of *V. vulnificus* for INT-407 cells. INT-407 cells were infected with the wild type (WT), the *aphB* mutant [*aphB* (pJH0311)], or the complemented strain [*aphB* (pHG0602)] at various MOI for 2 h (left) or at an MOI of 10 for various incubation times (right). The data are the means plus standard errors of the means (SEM) of results from three independent experiments.

in expression with a *P* value of ≤ 0.05 were considered to represent open reading frames regulated by AphB.

AphB is essential for cytotoxicity toward epithelial cells in vitro. Previously, we constructed an isogenic *aphB* mutant of *V. vulnificus*, JR312, in which the wild-type *aphB* gene on the chromosome was replaced with the Δ *aphB* allele (28). To complement the *aphB* mutation, pHG0602 was constructed by subcloning *aphB* amplified by PCR using primers APHB033F (5'-AAGAGCTCGATGTGTCAGGAAATATG-3') and APHB033R (5'-TAGGTACCCTACATCGTTAGTGGATG-3') into the broad-host-range vector pJH0311 (6).

Cytotoxicity is evaluated by the quantification of cytoplasmic lactate dehydrogenase (LDH) activity released by the damage of plasma membranes (31). The preparation of the INT-407 (ATCC CCL-6) human intestinal epithelial cells and infection with the *V. vulnificus* strains were performed as described previously (27). Monolayers of INT-407 cells infected with the wild type, the *aphB* mutant, and a complemented strain at different multiplicities of infection (MOI) were incubated for 2 h, and then the LDH activities in the supernatants were determined (Fig. 1). When the MOI was up to 10, cells infected with the *aphB* mutant JR312 exhibited significantly less LDH activity than those infected with the wild type. The level of LDH activity from the INT-407 cells infected with the *aphB* mutant was almost 10-fold lower than that from the cells infected with the wild type when the MOI was 5. Also, the LDH activities from INT-407 cells infected at an MOI of 10 were compared at different incubation times, as shown in Fig. 1. When incubated for as long as 4 h, the cells infected with the *aphB* mutant JR312 exhibited lower levels of LDH activity than the cells infected with the wild type. The lower levels of LDH activity were restored to levels comparable to those obtained from the cells infected with the wild type when the cells were infected with the complemented strain, JR312(pHG0602) (Fig. 1). Therefore, it was confirmed that the attenuated cyto-

toxic activity of the *aphB* mutant resulted from the inactivation of functional *aphB* rather than from any polar effects on genes downstream of *aphB*. These results suggest that AphB is essential for *V. vulnificus* to infect and injure host cells.

AphB is required for adhesion to epithelial cells and motility in vitro. For the adhesion assay, INT-407 cells seeded onto glass coverslips were infected at different MOIs for 1 h and then washed to remove nonadherent bacteria as described previously (16, 27). When INT-407 cells were infected at an MOI of 5, the wild-type strain adhered to the INT-407 cells and reached a concentration of 8 CFU per cell after 1 h of infection (Fig. 2A). The amount of adhered bacteria increased as the infection level (MOI) increased and reached about 46 CFU per cell at an MOI of 20 (Fig. 2A). In contrast, the *aphB* mutant JR312 was consistently and significantly less adherent than the wild type at all MOI tested. When cells were infected at an MOI of 10, the number of CFU of the *aphB* mutant per INT-407 cell was about twofold less than that of CFU of the wild type (Fig. 2A). The wild type and JR312(pHG0602) formed small clusters of aggregated bacteria on the INT-407 cell surfaces. With JR312, a much smaller area of the intestinal cell surface was covered with bacteria and no clusters of aggregated bacteria were observed (Fig. 2B). These results clearly revealed that the *aphB* mutant was significantly impaired in its ability to attach to the epithelial cells.

It was also noteworthy that light micrographs revealed that marked cellular damage such as cytoplasmic loss and nuclear-material condensation appeared in many Giemsa-stained INT-407 cells after infection with the wild type and JR312(pHG0602) (Fig. 2B). Fewer dead cells were observed after infection with the *aphB* mutant JR312. The cells infected with JR312 exhibited less surface damage and less cytoplasmic loss than those infected with the other strains. The general patterns of the abilities of the *V. vulnificus* strains to damage INT-407 cells

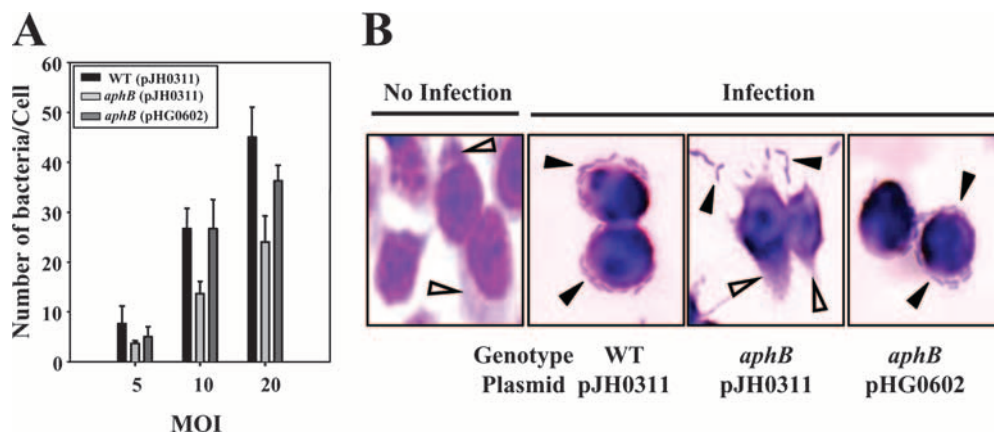


FIG. 2. Adhesion of the *V. vulnificus* strains. (A) INT-407 cells were infected at different MOI, as indicated. After incubation with the bacteria for 1 h, the INT-407 cells were rinsed to remove any nonadhering bacteria. Adherent bacteria were quantified and expressed as the number of bacteria per cell in the coverslip tissue cultures. WT, wild type; [*aphB* (pJH0311)], *aphB* mutant; [*aphB* (pHG0602)], complemented strain. (B) INT-407 cells were infected with the wild type, the *aphB* mutant, or the complemented strain at an MOI of 10 for 1 h as indicated and morphologically observed using a light microscope (original magnification, $\times 1,200$) after Giemsa staining. The adhered *V. vulnificus* cells (closed arrowheads) and the cytoplasm of the INT-407 cells (open arrowheads) are indicated.

were similar to the patterns of the cytotoxic activities as determined by measuring LDH activity (Fig. 1).

To determine whether or not AphB_v is required for motility, JR312 was tested for its ability to migrate on a semisolid plate surface with 0.3% agar compared to that of the wild type. As shown in Fig. 3, the growth of JR312 away from the inoculation point decreased compared to that of the wild type, and the diameter of the swimming area of the mutant was significantly reduced, to 20% of that of the wild type. The reintroduction of recombinant *aphB* substantially increased motility (Fig. 3), indicating that AphB is necessary for the optimum motility of *V. vulnificus*.

AphB is essential for virulence in mice. The 50% lethal doses (LD₅₀s) of the wild type and the *aphB* mutant were compared using ICR mice (specific-pathogen free; Seoul National University), as described elsewhere (27). The LD₅₀s of

the *V. vulnificus* strains for mice infected intraperitoneally were as follows: wild type, 1.3×10^1 CFU, and JR312, 3.9×10^4 CFU. Each inoculation group comprised six iron-overloaded mice, and inocula ranged from 10^0 to 10^8 CFU in 10-fold increments. All manipulations of mice were approved by the Institute of Laboratory Animal Resources of Seoul National University. The contrast in the LD₅₀s of the *aphB* mutant JR312 and the wild type showed the *aphB* mutant to be significantly less virulent than the wild type. This result indicates that AphB_v is essential for the virulence of *V. vulnificus* in mice as well as in tissue cultures. Thus, the results of the present study make it reasonable to conclude that the *aphB* gene is important for the pathogenicity of the bacterium.

Prediction and verification of the AphB regulon. By using a *V. vulnificus* whole-genome microarray, transcripts from the *aphB* mutant JR312 were compared to those from the wild

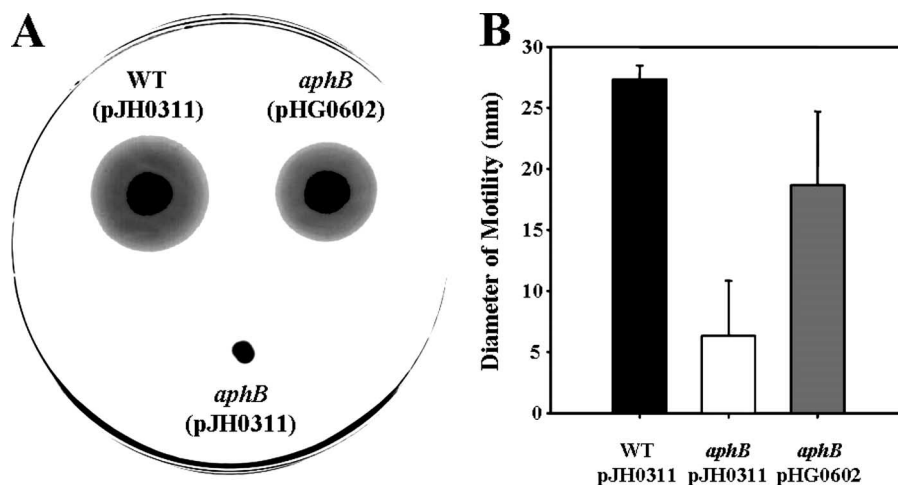


FIG. 3. Motilities of the *V. vulnificus* strains. (A) The areas of motility of the wild type (WT), the *aphB* mutant [*aphB* (pJH0311)], and the complemented strain [*aphB* (pHG0602)] grown for 18 h on plates with Luria-Bertani medium supplemented with 2.0% (wt/vol) NaCl and 0.3% agar were photographed by using a digital imaging system (UTA-1100; UMAX Technologies, Inc.). (B) The diameters of motility areas are the means plus SEM of results from three independent experiments.

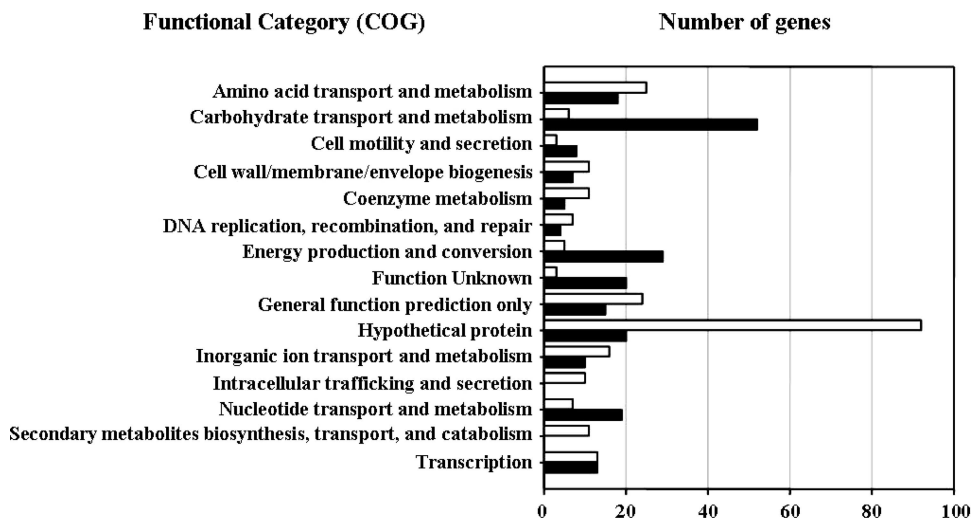


FIG. 4. Number of genes regulated by AphB. Genes with expression ratios of ≥ 2.828 on the basis of microarray analysis results were considered to be regulated by AphB. Functional categories (COG) corresponding to at least 10 genes are presented and are based on the database for the *V. vulnificus* CMCP6 genome, which was retrieved from GenBank (accession numbers AE016795 and AE016796). Closed and open bars represent the genes up-regulated and down-regulated by AphB, respectively.

type. The microarray screen predicted 489 genes potentially regulated by AphB, 230 of which were up-regulated and 259 of which were down-regulated. The predicted genes of the AphB regulon are distributed throughout the two chromosomes of *V. vulnificus*. A complete list of names or locus tags of the 489 genes is shown in Table S1 in the supplemental material. Although a substantial portion of the predicted genes are of hypothetical or unknown functions, some belong to functional categories (Fig. 4). Thirteen of these categories, including amino acid transport and metabolism, carbohydrate transport and metabolism, cell motility and secretion, cell wall/membrane/envelope biogenesis, coenzyme metabolism, DNA replication/recombination/repair, energy production and conversion, inorganic-ion transport and metabolism, intracellular trafficking and secretion, nucleotide transport and metabolism, secondary metabolite biosynthesis/transport/catabolism, and transcription, correspond to at least 10 genes (Fig. 4).

Most of the genes that we predicted to be part of the AphB

regulon on the basis of microarray analysis results were not previously reported to be AphB regulated. Therefore, AphB regulation of the newly predicted genes was experimentally verified using a quantitative real-time PCR (RT-PCR). cDNA was synthesized with a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA), and quantitative RT-PCR amplification of the cDNA was performed with a pair of primers (Table 1) as described previously (2). Sixteen genes, 10 of which were up-regulated and 6 of which were down-regulated by AphB, were randomly selected from the microarray data set, and the regulation of their transcription by AphB was examined. Quantitative RT-PCR revealed that AphB regulates the transcription of 14 of the 16 genes (Fig. 5), suggesting that most of the data set obtained from the microarray analysis is valid. One possible explanation for our inability to demonstrate AphB regulation of the other two genes is that the conditions used for either the microarray analysis or the quantitative RT-PCR were not optimal. Nevertheless, the results

TABLE 1. Oligonucleotides used for quantitative RT-PCR

Primer name	Oligonucleotide sequence (5'-3')		Locus tag ^a
	Forward	Reverse	
BACT001	ACAGGGCTTTCAGGCACAGAC	ACTCTTCCACCACCTCCGAAGCTG	VV1_1996
CADC001	ACTGCGTGATGGGCGAGAAG	GCTGGCTCCAACAATFCAACCTC	VV1_2058
CPAC001	AGTGGTGGTCTGTGTGGTGTG	GATAACGGAATCAGGCGGCTTTG	VV1_2331
FATB001	GCTATTGGTGATTGACGGTGATAAG	GACGGTCTCTTTGATGTGCGGAAAC	VV2_0842
FLAD001	TGAGATACCGTCGTTGGCGTTAC	GCAATGACAGCACAGCGTTACC	VV1_0214
FRU001	TCGGTTTGACGCCCTCTTCG	CTTCAATGGACGGCATFCGCTTAC	VV2_1357
GGDEF001	AGTTCTCGCTCCGCCATCTTG	TGCCGACAACCCATTAGTGACC	VV2_1319
GLU001	GAACACCACCAGCAACGACAAC	GGCGGTAAGCAAACATCACAGG	VV1_2999
HUPA001	GCTCTACTTGATTGCGGTCCG	ACTGTAGGTGAAGATGGCGAAGG	VV2_0276
MAL001	CTACAAGAAGTGCCTGCCAAGTG	GACTGCGTAAGAGCCAATCACAC	VV2_1250
NANA001	GCAATCGCTTTTCGCTCTTCAAC	GATCGCCGCTCCTCATACACC	VV2_0730
OMPC001	AGCCCAGAAATCGTCGCCTTC	GCCCAATCTAACACCATCCGTTG	VV1_0238
SECE001	TGTATGGTGAACCTTCTGTGGTATTC	GCATAGTTTCTTGCGGAGTAGGC	VV1_1205
VENB001	TCGGTCTGTCCGATGTTGGTTAG	GCCAGATTCATCGCCAGTTGTTG	VV2_0838

^a Locus tags are based on the database for the *V. vulnificus* CMCP6 genome, which was retrieved from GenBank (accession numbers AE016795 and AE016796).

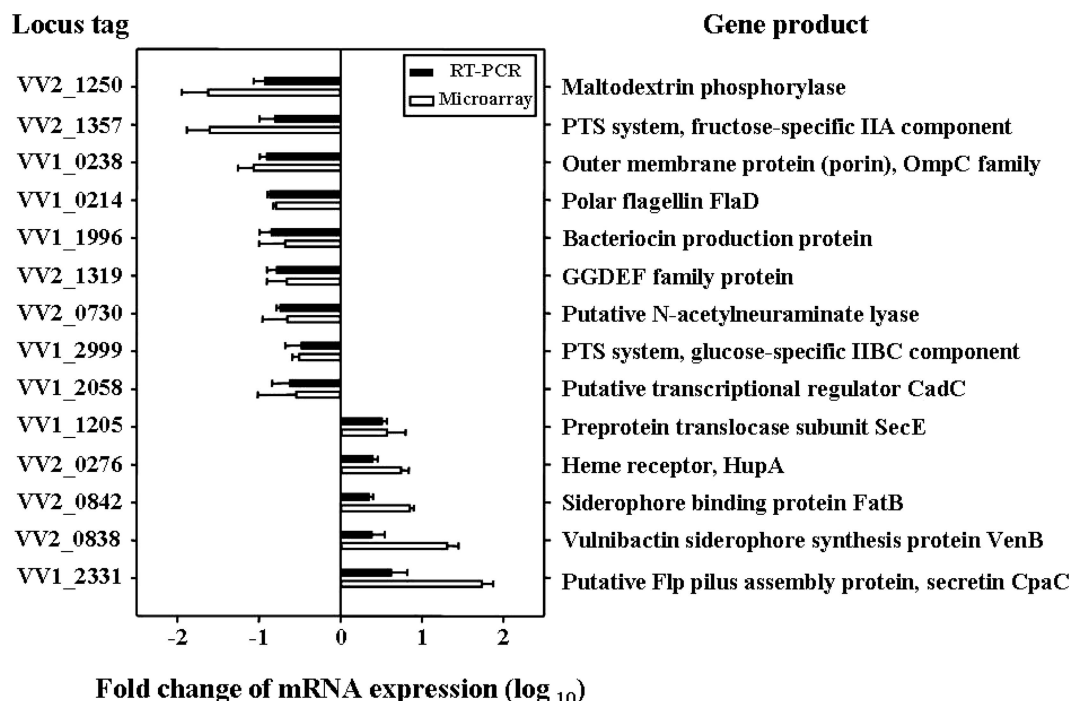


FIG. 5. Verification of the newly predicted genes as part of the AphB regulon. Fourteen genes from the pool of AphB regulon members newly predicted on the basis of microarray analysis results were analyzed by RT-PCR. Each column represents the mRNA expression level in the *aphB* mutant relative to that in the wild type. Averages and SEM were calculated from the results of at least three independent experiments. Locus tags are based on the database of the *V. vulnificus* CMCP6 genome as described in the legend to Fig. 4, and the products of the 14 genes are listed on the right. PTS system, phototransferase system.

suggest that a significant portion of the AphB regulon predicted on the basis of microarray analysis results is indeed regulated by AphB and that AphB is a global regulator in *V. vulnificus*.

These results provide the first direct evidence that AphB is essential for the virulence of bacterial pathogens in which TCP and CT are not found. However, transcript profiles of the *aphB* mutant revealed that virulence factors that directly injure host cells, such as hemolysin, elastolytic protease, and RtxA, are not regulated by AphB (see Table S1 in the supplemental material) (15, 23, 32). Rather, many genes involved primarily in the metabolism and utilization of nutrients were identified as members of the AphB regulon (Fig. 4 and 5; see Table S1 in the supplemental material). Fifty-eight genes, including genes encoding maltodextrin phosphorylase, a fructose-specific IIA component, a glucose-specific IIBC component, and a putative N-acetylneuraminase lyase, are classified into the Clusters of Orthologous Groups (COG) category of carbohydrate transport and metabolism, and 43 genes are classified into the COG category of amino acid transport and metabolism (Fig. 4 and 5; see Table S1 in the supplemental material). Additionally, genes involved in the uptake of iron, such as *fatB*, *hupA*, and *venB*, are also regulated by AphB (Fig. 5) (1, 17, 18). These results suggest that AphB regulates numerous genes involved in the acquisition and metabolism of nutrients and aids growth and adaptation in host environments, potentially contributing to the overall success of *V. vulnificus* in pathogenesis.

The motility of the pathogenic bacteria is essential for a successful infectious process because it facilitates adhesion to

and the colonization of host epithelial cells (24). The *aphB* mutant was clearly impaired in adherence and motility (Fig. 2 and 3), and many genes known to be potentially important for the motility of bacteria were identified as members of the AphB regulon (Fig. 4 and 5; see Table S1 in the supplemental material). Besides *flaD* (Fig. 5), 11 genes involved in the biosynthesis, assembly, and transport of flagellum proteins are apparently regulated by AphB (Fig. 4; see Table S1 in the supplemental material). Altering the physiochemical characteristics of the *V. vulnificus* cell surface has also been postulated to modify the relative adhesive properties of the bacterium (25, 26, 27). Consistent with this possibility, 18 genes including *cpaC*, encoding the putative Flp pilus assembly protein classified into the COG category of cell wall/membrane/envelope biogenesis, appeared to be regulated by AphB (Fig. 4 and 5; see Table S1 in the supplemental material). Additionally, a protein with a GGDEF domain is regulated by AphB (Fig. 5). Proteins with GGDEF domains are predicted to control the cellular level of a second messenger, the cyclic di-GMP, and thus regulate bacterial cell surface adhesiveness (3). Undoubtedly, adhesion to intestinal epithelial cells is an important step in the disease process of *V. vulnificus* infection. However, the exact role of AphB in the pathogenicity of *V. vulnificus* needs additional study.

Microarray data accession number. All primary microarray data are available from CIBEX at <http://cibex.nig.ac.jp/cibex2/index.jsp> under accession number CBX42.

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