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Identification and Characterization of the *Vibrio vulnificus* Phosphomannomutase Gene

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Abstract Numerous virulence factors such as O antigen have been proposed to account for the fulminating and destructive nature of *V. vulnificus* infections. To better characterize the role of O antigen, a *pmm* gene encoding a phosphomannomutase was identified and cloned from *V. vulnificus*. The deduced amino acid sequence of the *pmm* was 42 to 71% similar to that reported from other Enterobacteriaceae. Functions of the *pmm* gene in virulence were assessed by the construction of an isogenic mutant, whose *pmm* gene was inactivated by allelic exchanges, and by evaluating its phenotype changes *in vitro* and in mice. The disruption of *pmm* resulted in a loss of more than 90% of phosphomannomutase, and reintroduction of recombinant *pmm* could complement the decrease of phosphomannomutase activity, indicating that the *pmm* gene encodes the phosphomannomutase of *V. vulnificus*. There was no difference in the LD_{50}s of the wild-type and the *pmm* mutant in mice, but the LD_{50}s observed by the mutant complemented with recombinant *pmm* were lower. Therefore, it appears that PMM is less important in the pathogenesis of *V. vulnificus* than would have been predicted by examining the effects of injecting purified LPS into animals, but it is not completely dispensable for virulence in mice.

Key words: *V. vulnificus*, *pmm*, LPS, virulence

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of food-borne diseases such as life-threatening septicemia and possibly gastroenteritis in individuals with underlying predisposing conditions such as liver damage, excess levels of iron, and immunocompromised conditions [2, 14, 15]. Wound infections result from exposure to seawater or from the handling of shellfish contaminated with *V. vulnificus*. Mortality from septicemia is very high (>50%), and death may occur within one to two days after the first signs of illness [2, 14].

The symptoms which occur with *V. vulnificus* septicemia, as well as the inflamatory response observed in wound infections, are those associated with the endotoxic activity of lipopolysaccharide (LPS) molecules, rendering it a prime candidate as a major virulence factor [21]. Very little is known so far regarding the biosynthetic pathway for the LPS of *V. vulnificus*, and its genes encoding enzymes involved in the production of LPS have not yet been identified. However, it is generally believed that a similar biosynthetic pathway has been proposed to operate in Gram-negative bacteria. The molecular genetics of LPS production of *Pseudomonas aeruginosa* have been widely studied [17]: The glycolytic intermediate fructose-6-phosphate is first converted to mannose-6-phosphate by the action of phosphomannose isomerase. Mannose-1-phosphate is then formed by the action of phosphomannomutase (PMM) and further converted to GDP-mannose, which is a common metabolite used for the synthesis of different saccharides of LPS [16].

The characteristics of *V. vulnificus* LPS as a potential virulence factor have been studied primarily using the purified LPS in animal models. In fact, McPherson *et al.* [18] demonstrated that intravenous injections of *V. vulnificus* LPS into mice caused mean arterial pressure to decrease within 10 min, with a further decline and subsequent death in 30 to 60 min. One of the leading factors believed to contribute to fatality in Gram-negative septicemia is the overproduction of tumor necrosis factor (TNF), leading to overstimulation of nitric oxide synthase, in response to LPS. In fact, increased mortality in cirrhotic mice due to *V. vulnificus* infection is dependent on an *in vivo* TNF-α response [6]. Purified *V. vulnificus* LPS causes severe hypotension and death within 1 h, when injected into rats [5]. However, when a nitric oxide synthase inhibitor is administered to rats along with LPS, the lethality of

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Table 1. Plasmids and bacterial strains used in this study.

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
V. vulnificus		
ATCC29307	Clinical isolate; virulent	Laboratory collection
HS01	ATCC29307 with *luxS::nptI*; Ap^s, Km^r	This study
JH001	ATCC29307 with *pmm::nptI*; Ap^s, Km^r	This study
E. coli		
DH5α	*supE44 Δ lacU169 (φ80 lacz* ΔM15*) hsdR17 recA1 endA1* gyrA96 thi-1 relAI	Laboratory collection
SY327 λ *pir*	*Δ(lac pro) argE*(Am) *recA56 gyrA rpoB* λ*pir*; host for π-requiring plasmids; conjugal donor	[20]
SM10 λ *pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*; Km^r; host for π-requiring plasmids; conjugal donor	[20]
Plasmids		
pGEM-T Easy	PCR product cloning vector; Ap^r	Promega
pUC18	Cloning vector; Ap^r	Laboratory collection
pBR322	Cloning vector; Ap^r, Tr^r	Laboratory collection
pUC4K	pUC4 with *nptI*; Ap^r, Km^r	[22]
pCVD442	R6K γ*ori*; sacB; Suicide vector; *oriT* of RP4; Ap^r	[4]
pRK415	IncP *ori*; broad-host-range vector; *oriT* of RP4; Tc^r	[12]
pKC600	494-bp PCR product containing *pmm* cloned into pGEM-T Easy vector; Ap^r	This study
pJH0020	Cosmid library containing *pmm*; Tc^r	This study
pJH0021	7.0-kb *Pst*I fragment containing *pmm* cloned into pUC18; Ap^r	This study
pJH0027	pBR322 with *pmm*, Ap^r, Tr^s	This study
pJH0028	pJH0027 with *pmm::nptI*; Ap^r, Km^r	This study
pJH0029	pCVD442 with *pmm::nptI*; Ap^r, Km^r	This study
pJH0030	pRK415 with *pmm*; Tc^r	This study

[a]: Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; Tc^s, tetracycline sensitive.

the endotoxin is reversed [18]. These data suggest that the stimulation of TNF, followed by nitric oxide (NO) production, leads to endotoxic shock in response to *V. vulnificus* LPS.

However, in contrast to the reported effects of administrating *V. vulnificus* LPS in experimental animals, a definitive analysis of the role of the *V. vulnificus* LPS by the construction of a defined mutation has not yet been reported. We therefore undertook this study to identify the function of the LPS during an infectious process, rather than the artificial system of injecting purified LPS, by constructing the isogenic PMM mutant of *V. vulnificus* and applying the molecular version of Koch's postulates [7].

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains used for plasmid DNA replication or conjugational transfer of plasmids were grown in Luria-Bertani (LB) broth or on LB broth containing 1.5% (w/v) agar. Unless otherwise noted, *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS). Cultures of *V. vulnificus* strains were grown at 30°C with aeration. Five ml samples were removed at log phase for determination of cell densities, PMM activities, and cellular protein concentrations. The PMM activities were determined according to the method of Goldberg *et al.* [9]. One unit of the enzyme activity was defined as the amount of enzyme needed to reduce 1 μmol of NADP to NADPH per min, as described previously [26]. Protein concentrations were determined by the method of Bradford [3], with bovine serum albumin as the standard. Averages and standard errors of the mean (SEM) were calculated from at least three independent determinations.

Cloning and Sequence Analysis of the *pmm* Gene

Recent reports suggest that environmental cues, such as cell density, may govern virulence gene expression in many Gram-negative bacteria [19]. It has been recognized that bacteria can regulate gene expression in response to cell density, and quorum-sensing systems based on acylhomoserine lactone autoinducer (AI-1) molecules are widespread among Gram-negative bacteria [8]. Recently, an alternative, highly conserved quorum-sensing system used by both Gram-positive and Gram-negative bacteria has been identified [1]. This system requires a highly conserved locus, *luxS*. It has been proposed that LuxS acts on a metabolic intermediate to synthesize a unique signaling molecule, termed autoinducer 2 (AI-2). However, until now, no study addressed the genes whose expression is regulated by the AI-2 quorum sensing system in *V. vulnificus*.

Therefore, as an effort to identify genes that are regulated by the AI-2 quorum-sensing system, we cloned genes differentially

expressed by a *luxS* mutant, HS01, using DD (differential display) PCR [25]. The *luxS* mutant was constructed by allelic exchange in ATCC29307 (Choi, 2002, unpublished data). Cultures of ATCC29307 and the *luxS* mutant were grown to log phase, and total cellular RNAs were isolated using a Trizol reagent kit according to the manufacturer's specifications (GIBCO-BRL, Gaithersburg, MD, U.S.A.). For reverse transcriptase (RT) PCR of the genes differentially expressed in *luxS* mutant, total RNA was reverse transcribed essentially following the manufacturer's (GIBCO-BRL) protocol in a total volume of 20 ml reaction mixture. For PCR amplification, each 2 μl sample of the resulting cDNA was used as a template, and OPA4 (5'-AATCGGGCTG-3') as a random primer.

The resulting PCR product, a 494-bp DNA fragment, was purified from the agarose gels and cloned into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) according to the manufacturer's specifications, yielding plasmid pKC600. Since the deduced amino acid sequence of the resulting PCR product revealed 79% identity with that of *V. cholerae pmm*, the DNA was labeled with [α-^{32}P]dCTP and named PmmP. To clone the full gene of the *pmm*, a cosmid library of *V. vulnificus* ATCC29307, constructed using pLAFR3 [13, 24], was screened using PmmP as a probe. A colony showing a positive signal was isolated, and the cosmid DNA was purified and named pJH0020 (Fig. 1). A 7.0-kb band from the cosmid DNA digested with *Pst*I was purified and ligated into pUC18 to result in pJH0021, as shown in Fig. 1.

The nucleotide sequences of the DNA fragments in pJH0021 were determined by primer walking (Korea Basic Science Research Center, Kwang-Ju, Korea), and the sequence data were submitted to the GenBank [Accession number AY205154]. The nucleotide sequence revealed a coding region consisting of 1,707 nucleotides (Fig. 1). The *pmm* from *V. cholerae* was 61% identical in nucleotide sequences with the coding region in pJH0021 (data not shown). This information proposed that the coding region

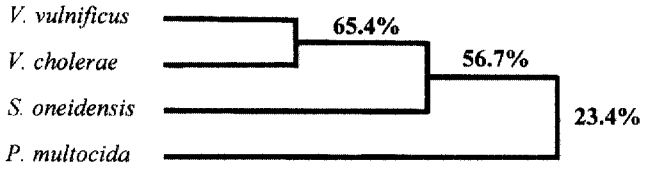

Fig. 2. Amino acid-sequence relatedness of PMMs of *V. vulnificus* and other bacteria.
Dendrogram showing relatedness of PMM was derived using the DNASIS alignment program (version 2.6, Hitachi Software Engineering Co., Ltd., Japan), and based on the amino acid sequences in the GenBank databases (NCBI).

is a homolog of the *pmm* gene from *V. cholerae* and led us to name the coding region *pmm* of *V. vulnificus*. The amino acid sequence deduced from the *pmm* nucleotide sequence revealed a protein, a PMM composed of 568 amino acids with a theoretical molecular mass of 62,582 Da and a PI of 5.67. A database search for amino acid sequences similar to those deduced from the *pmm* coding region revealed three other PMM proteins from *V. cholerae*, *Shewanella oneidensis*, and *Pasteurella multocida* strains with high levels of identity (Fig. 2, http//www.ncbi.nml.nih.gov). The amino acid sequence of the *V. vulnificus* PMM was 71% identical to that of the PMM of *V. cholerae*, and their identity appears evenly throughout the whole proteins (data not shown). All of this information confirmed that the *pmm* gene encodes the PMM of *V. vulnificus*.

Generation and Confirmation of the *pmm::nptI* Mutant

The *pmm* gene in pJH0027 (Table 1) that was constructed by ligation of a 1.7-kb *Bam*HI-*Hin*dIII fragment of pJH0021 with pBR322 was inactivated *in vitro* by insertion of *nptI* encoding for aminoglycoside 3'-phosphotransferase and conferring resistance to kanamycin [22]. The 1.2-kb DNA fragment carrying *nptI* was isolated from pUC4K (Pharmacia, Piscataway, NJ, U.S.A.) and inserted into a unique *Nsi*I site present within the open reading frame (ORF) of *pmm*. The 2.9-kb *pmm::nptI* cartridge from the resulting construct

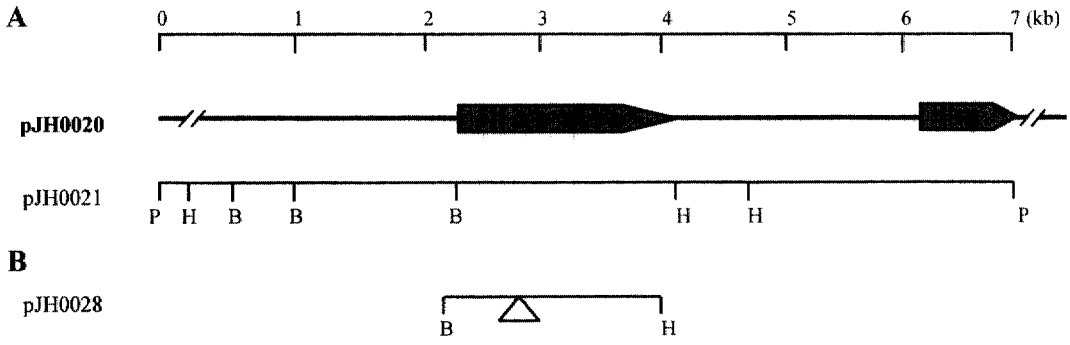

Fig. 1. Physical map of the *V. vulnificus pmm* gene on the plasmids used in this study.
(A) Plasmid pJH0021 was used to determine the nucleotide sequence of *pmm*. The shaded boxes represent the locations of one complete open reading frame (*pmm*) and partial open reading frame (*alzC*) and directions of their transcription. (B) Depicted is the region cloned in the plasmid used for construction of *pmm::nptI* mutant. The insertion position of *nptI* cassette is indicated by the open triangle. Abbreviations; P, *Pst*I; B, *Bam*HI; H, *Hin*dIII.

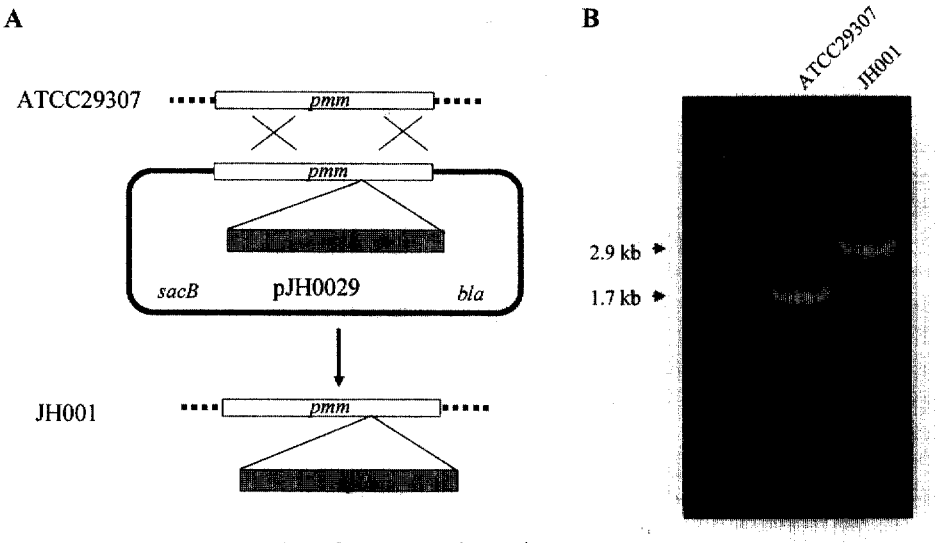

Fig. 3. Allelic exchange procedure and construction of *pmm::nptI* isogenic mutant.
(A) Double homologous recombinations between strain ATCC29307 and plasmid pJH0029 led to interruption of *pmm* gene and resulted in construction of the mutant JH001. Dashed lines represent the bacterial chromosome; a full line, the plasmid DNA; open box, the target *pmm* gene; shaded box, the *nptI* gene; large X's represent genetic crossing over. Abbreviations; *SacB*, levansucrase gene; *bla*, β-lactamase gene. (B) PCR analysis of ATCC29307 and isogenic mutant generated by allelic exchange. Molecular size markers (1 kb plus DNA ladder, GIBCO-BRL) and PCR products are indicated.

(pJH0028, Fig. 2B) was liberated and ligated with *Sma*I-digested pCVD442 [4], forming pJH0029 (Table 1).

E. coli SM10*pir* (containing pJH0029) was used as a conjugal donor to generate the *pmm::nptI* mutant of *V. vulnificus* ATCC29307 by homologous recombination (Fig. 3A). The *V. vulnificus pmm* mutant chosen for further analysis was named JH001. The conjugation and isolation of the transconjugants were conducted using the methods previously described [10], and a double crossover, in which each wild-type *pmm* gene was replaced with the *pmm::nptI* allele, was confirmed by PCR as shown in Figure 3B. PCR analysis of genomic DNA from ATCC29307 with primers PMM002 (5'-GTACTGCAGTTTAGAGAAAACAGAG-AGAGTAAG-3') and PMM003 (5'-TTAGAATTCAAG-AGCACACTCATACGTCGAGTG-3') produced a 1.7-kb fragment (Fig. 3B), whereas genomic DNA from the JH001 resulted in an amplified DNA fragment, approximately 2.9-kb in length. The 2.9-kb fragment is in agreement with the projected size of the DNA fragment containing wild-type *pmm* (1.7-kb) and the *nptI* gene (1.2-kb). The inserted *nptI* DNAs were stably maintained in the mutant, as determined by maintenance of kanamycin resistance (all of more than 500 colonies tested) and by generation of the appropriate-sized DNA fragment by PCR (data not shown).

Effects of Mutation in *pmm* Gene on Phosphomannomutase Activity

For ATCC29307, PMM was produced and reached a maximum 45.0 units (Fig. 4). The disruption of *pmm* in the mutant JH001 resulted in reduced production of PMM activity ($p<0.05$). The residual level of PMM activity in JH001 corresponded to approximately one-tenth of that in the wild-type. These data demonstrated that the *pmm* gene encoded the PMM of *V. vulnificus*. However, the PMM activity in JH001 was still evident and its level was significantly higher than the background level observed, when control assay was carried out in the absence of mannose. This observation that the mutant still exhibited PMM activity indicated the existence of at least one more PMM (or its homolog) being produced by *V. vulnificus* ATCC29307.

To rule out the possibility that the decrease of PMM activity by more than 90% resulted from polar effects of the *pmm*

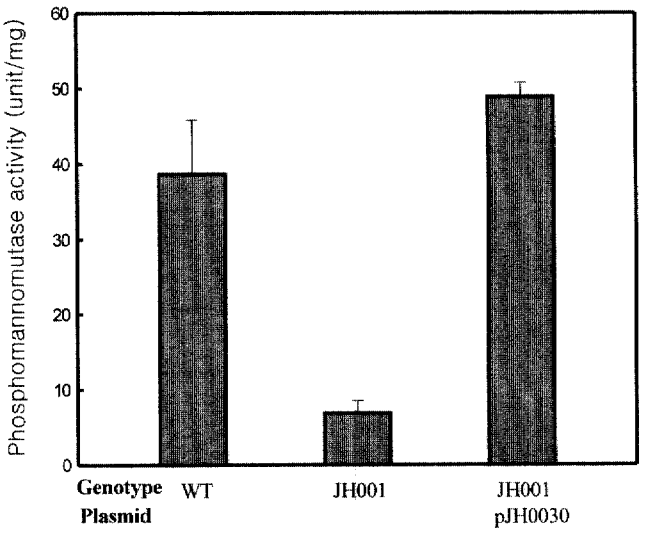

Fig. 4. PMM activities of ATCC29307 and isogenic *pmm* mutant.
Complementation of mutant with functional *pmm* (pJH0030) was also presented as indicated. Error bars represent the S.E.M., WT, wild-type.

insertional mutation on downstream genes, we examined if reintroduction in pJH0030 carrying the recombinant *pmm* could complement the decrease of PMM activity of JH001 cells. For this purpose, pJH0030 was constructed by subcloning the *pmm* amplified by PCR using primers PMM002 and PMM003 and then digested with *Pst*I and *Eco*RI into the broad host-range vector pRK415 [12] linearized with the same enzymes. The resulting plasmid was mobilized into *V. vulnificus* by conjugation. The PMM activity of the JH001(pJH0030) was restored to a level comparable to, even higher than, the wild-type level of ATCC29307 (Fig. 4). Therefore, the decreased PMM activity of JH001 resulted from inactivation of functional *pmm* rather than any polar effects on any genes downstream of *pmm*.

Effects of *pmm* Mutation on the Virulence of *V. vulnificus*

We examined the virulence of *V. vulnificus* ATCC29307 and JH001, using both iron dextran-treated and normal ICR mice (Specific Pathogen-Free; Daehan Animal Co, Taejon, Korea), as described [11]. Briefly, female mice from 7 to 11 weeks of age were housed under specific pathogen-free conditions. For the determination of LD_{50}, bacteria grown in BHI-N [11] broth overnight at 25°C were harvested and suspended in PBS to appropriate concentrations, ranging from 10^1 to 10^7 CFU in 10-fold increments. Group of (n=6) 7-weeks-old normal female mice were injected subcutaneously with 0.1 ml of serial dilutions of bacterial suspensions. The infected mice were observed for 24 h, and the LD_{50} were calculated by the method of Reed and Muench [23]. For experiments involving iron treatment, mice were injected intraperitoneally with 250 μg of iron dextran per g of body weight immediately before injection with bacterial cells.

As shown in Table 2, there was no difference in the LD_{50}s of ATCC29307 and JH001 in normal mice (none-iron-treated), which were as high as 10^4 CFU for both strains. In iron dextran-treated mice, the LD_{50}s observed by both strains were also indistinguishable (Table 2). Therefore, in the mouse model of subcutaneous infection, in which LD_{50}s were compared with wild-type and *pmm* mutant strains, the PMM appeared to be not important for disease. However, it is notable that LD_{50}s observed by JH001(pJH0030) were lower in both normal mice and iron dextran-treated mice. Taking these results together, it is reasonable to assume that the PMM is not completely dispensable for virulence in mice; however, we cannot make any conclusions as to the role of PMM in general.

Table 2. Effects of the *pmm* mutation on the lethality of *V. vulnificus* to mice[a].

	Bacterial lethality (LD_{50})[c]	
Strain	Non-iron-treated mice	Iron-treated mice
Wild-type	6.46×10^4	1.22×10^3
JH001	8.41×10^4	2.31×10^3
JH001(pJH0030)	9.70×10^2	2.65×10^2

[a,b]: Details are in the text.

The major problem to be addressed is the difference in virulence between the wild-type and the JH001 complemented with pJH0030. Although other explanations are possible, this discrepancy could be related to the level of PMM expressed in the cells during infection. Complementation with pJH0030 would provide JH001 with *pmm*, which is expressed under the P_{tac} promoter of pRK415 [12]. Therefore, it might be possible that the number of PMM molecules in the complemented cells could be relatively higher than that in wild-type cells, in which all PMM molecules are products of *pmm*, which is expressed by its own promoter carried on the chromosome. This possibility strongly underscores the necessity of understanding the regulation of *pmm* expression. However, no promoter(s) of the *pmm* gene has been identified, and the molecular mechanisms by which the bacterium modulates the expression of the *pmm* gene have not yet been characterized. This lack of information on the regulatory characteristics of the *pmm* gene makes it difficult to understand how the expression pattern and level of PMM varies spatially and temporally during infection with viable *V. vulnificus*.

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