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The *pyrH* Gene of *Vibrio vulnificus* Is an Essential In Vivo Survival Factor▽

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We have suggested an important role of the *pyrH* gene during the infectious process of *Vibrio vulnificus*. Previously, we have identified 12 genes expressed preferentially during human infections by using in vivo-induced antigen technology. Among the in vivo-expressed genes, *pyrH* encodes UMP kinase catalyzing UMP phosphorylation. Introduction of a deletion mutation to the *pyrH* gene was lethal to *V. vulnificus*, and an insertional mutant showed a high frequency of curing. We constructed a site-directed mutant strain (R62H/D77N) on Arg-62 and Asp-77, both predicted to be involved in UMP binding, and characterized the R62H/D77N strain compared with the previously reported insertional mutant. We further investigated the essential role of the *pyrH* gene in the establishment of infection using the R62H/D77N strain. Cytotoxicity was decreased in the R62H/D77N strain, and the defect was restored by an in *trans* complementation. The intraperitoneal 50% lethal dose of the R62H/D77N strain increased by 26- and 238,000-fold in normal and iron-overloaded mice, respectively. The growth of the R62H/D77N strain in 50% HeLa cell lysate, 100% human ascitic fluid, and 50% human serum was significantly retarded compared to that of the isogenic wild-type strain. The R62H/D77N mutant also had a critical defect in the ability to survive and replicate even in iron-overloaded mice. These results demonstrate that *pyrH* is essential for the in vivo survival and growth of *V. vulnificus* and should be an attractive new target for the development of antibacterial drugs and replication-controllable live attenuated vaccines.

Vibrio vulnificus is an estuarine bacterium that opportunistically infects a human being through the consumption of contaminated seafood or wound infection. *V. vulni*ficus septicemia preferentially occurs in patients with underlying hepatic diseases or other immunocompromised conditions and results in a rapid progress and high mortality rate of >50% (10, 22, 35). During the infectious process, the *V. vulnificus* is confronted with dramatic environmental changes, and the bacteria seem to cognitively sense the changes in the host milieu. For the successful infection, *V. vulnificus* should establish coordinated spatiotemporal expression of various virulence genes in vivo (11, 12). Our group has previously reported 12 in vivo expressed genes by using in vivo-induced antigen technology (17). Among them, *pyrH* encodes UMP kinase, which catalyzes phosphorylation of UMP to UDP (24, 26). It was reported that UMP kinase senses the environmental pyrimidine pool and directly regulates pyrimidine-specific CarP1 promoter of carbamoylphosphate synthetase of *Escherichia coli* responsible for the early stage de novo synthesis of pyrimidines (15). Klarsfeld et al. have reported that *pyrE* of *Listeria monocytogenes*, another de novo pyrimidine biosynthetic gene, preferentially expressed the intracellular milieu of host cells through a transposon mutant library screening experiment (18). These previous reports suggest that limited availability of pyrimidines in animal tissues might be responsible for the in vivo induction of the enzymes required for de novo biosynthesis of them (8, 23).

In order to further characterize the *pyrH* gene, we have tried to construct *pyrH* gene-specific mutant strains. Previously, we have constructed an insertional *pyrH* mutant and showed a significant decrease in virulence (17). Unfortunately, the insertional mutations showed a high frequency of curing, especially when the mutant was introduced to mice. Furthermore, after massive preliminary experimental trials, we concluded that introduction of a deletion mutation on the *pyrH* gene is lethal to *V. vulnificus*. Therefore, in order to verify the role of the *pyrH* gene during the infectious process, we decided to compromise enzymatic activity of PyrH by introducing site-directed mutations on the *pyrH* rather than by abolishing the gene. Bucurenci et al. have elucidated critical amino acid residues of *E. coli* PyrH by using genetic and biochemical assays (3). Recently, molecular structure of PyrH encoded by *E. coli* and *Pyrococcus furiosus* was solved (2, 24). In the present study, we introduced site-directed mutations on the chromosomal *pyrH* residues of Arg-62 and Asp-77, both involved in UMP binding, based on the comparison of sequence homologies with the *E. coli pyrH*. Then we investigated the characteristics of the site-directed mutated strain (R62H/D77N) and proved essential role of PyrH in the survival and growth of *V. vulnificus* in vivo.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
V. vulnificus		
MO6-24/O	Clinical isolate	28
CMM1474	MO6-24/O with site-directed mutation on R62H/D77N	This study
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Laboratory collection
SY327 λ*pir*	Δ(*lac pro*) *argE*(Am) *rif nalA recA56* λ*pir* lysogen	25
SM10 λ*pir*	*thi thi leu tonA lacY supE recA*::RP4-2-Tc^r::Mu λ*pir* R6K lysogen; Km^r	25
ER2566	F$^-$ λ$^-$ *fhuA2* [*lon*] *ompT lacZ*::T7 gene1 *gal sulA11* Δ(*mcrC-mrr*)*114*::IS*10* R(mcr-73::miniTn*10*-TetS)2 R(zgb-210::Tn*10*)(TetS) *endA1* [*dcm*]	New England Biolabs, Inc.
Plasmids		
pDM4	Suicide vector with ori R6K *sacB*; Cm^r	25
pLAFR3	IncP cosmid vector; Tc^r	34
pRK2013	IncP Km^r Tra Rk2$^+$ repRK2 *repE1*	7
pCR2.1-TOPO	PCR cloning vector	Invitrogen
pTYB12	N-terminal fusion expression vector in which the N terminus of a target protein is fused to an intein tag; Ap^r	New England Biolabs, Inc.
pCMM1426	pCR2.1-TOPO containing *pyrH* ORF	This study
pCMM1442	pCR2.1-TOPO containing *pyrH* ORF with R62H site-directed mutation	This study
pCMM1452	pCR2.1-TOPO containing *pyrH* ORF with R62H/D77N site-directed mutation	This study
pCMM1474	pDM4 containing *pyrH* ORF with R62H/D77N site-directed mutation	This study
pCMM1456	pTYB12 containing *pyrH* ORF	This study
pCMM1496	pTYB12 containing *pyrH* ORF with R62H site-directed mutation	This study
pCMM1498	pTYB12 containing *pyrH* ORF with D77N site-directed mutation	This study
pCMM1500	pTYB12 containing *pyrH* ORF with R62H/D77N site-directed mutation	This study
pCMM1486	pLAFR3 with *pyrH* ORF	This study

[a] Cm^r, Cm resistance; Tc^r, Tc resistance; Ap^r, Ap resistance; Km^r, Km resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids are listed in the Table 1. *E. coli* strains and *V. vulnificus* strains were grown in Luria-Bertani (LB) and in 2.5% NaCl heart infusion (HI) medium, respectively. Antibiotics were used at the following concentrations: for *E. coli*, ampicillin (Amp) at 100 μg/ml, kanamycin (Km) at 50 μg/ml, chloramphenicol (Cm) at 30 μg/ml, and tetracycline (Tc) at 12.5 μg/ml were used. For *V. vulnificus*, Amp at 20 μg/ml, Cm at 2 μg/ml, and Tc at 2 μg/ml were used. DNA manipulations were performed as previously described (31) and in accordance with the recommendations of manufacturer.

Construction of site-directed *pyrH* mutant. The chromosomal *V. vulnificus pyrH* site-directed mutant (R62H/D77N) was constructed by PCR-based mutagenesis and allelic exchange using the suicide vector pDM4 (25). In order to construct PCR template for the site-directed mutagenesis, a PCR-amplified fragment from MO6-24/O genomic DNA using the EP-pyrH-1 (GAAGATCT TCCTTAGAGATTGTGCAAAGATTAG, with the BglII site underlined) and EP-pyrH-2(GCTCTAGAAGCTTAGGCGGTAATTAGCGTACC, with the XbaI site underlined) primers was cloned into pCR2.1-TOPO plasmid (Gibco/Invitrogen, Co., Carlsbad, CA), yielding pCMM1426. Site-directed mutagenesis was performed by using the *Pfu* polymerase (Stratagene, La Jolla, CA) in accordance with the manufacturer's protocol. Primary R62H substitution on the *pyrH* gene was generated by PCR using pCMM1426 as the template with the primers R62H-for (GGTAACTTGTTCCATGGTGCTGGTCTAGC, with the NcoI site underlined) and R62H-rev (GCTAGACCAGCACCATGGAACAAGTTACC, with the NcoI site underlined). The plasmid harboring *pyrH* with R62H substitution was designated pCMM1442. The R62H substitution was screened by restriction mapping since the mutated allele should have a new NcoI site in the open reading frame (ORF). The substitution was further confirmed by DNA sequencing. Secondary D77N substitution was introduced similarly to pCMM1442 using the D77N-for (CGTGTTGTGGGTAACCACATGGG TATG) and D77N-rev (GCATACCCATGTGGTTACCCACAACAC) primers, and the resulting plasmid with both R62H and D77N substitutions was designated pCMM1452. A BglII-XbaI fragment from pCMM1452, which contained the R62H/D77N mutation, was subcloned to pDM4 yielding pCMM1474. The resulting suicide plasmid pCMM1474 was transformed into *E. coli* SM10 λ*pir* (33). The plasmid was transferred to *V. vulnificus* MO6-24/O by conjugation, and the transconjugants were selected on thiosulfate citrate bile sucrose agar plates containing Cm. The transconjugants were plated onto a 2.5% NaCl HI agar plate containing 10% sucrose to select clones that have undergone the second homologous recombination event forcing excision of the vector sequence and leaving only a mutated or wild-type allele of the gene. The mutated R62H/D77N allele on the chromosome was amplified by PCR and confirmed by the restriction mapping and DNA sequencing as described above.

PyrH enzyme activity assay. A 726-bp fragment containing the ORF of Vv-*pyrH* was PCR amplified from MO6-24/O genomic DNA by using the *pyrH*-start (CGGAATTCATGACGACTAACCCTAAACCAG, with the EcoRI site underlined) and *pyrH*-stop (TCCCCCGGGTTAGGCGGTAATTAGCGTACCTTC, with the SmaI site underlined) primers. The PCR product was cloned into pTYB12 (New England Biolabs, Inc., Beverly, MA), yielding pCMM1456. The pCMM1456 plasmid was transformed into *E. coli* ER2566 (New England Biolabs) by electroporation. An intein-Vv-PyrH fusion protein was induced by 0.5 mM 5-bromo-indolyl-3-chloro-isopropyl-β-D-galactopyranoside (IPTG). To prepare a bacterial lysate for affinity column chromatography, the pellet was resuspended in a lysis buffer (20 mM Tris-Cl [pH 7.5], 500 mM NaCl, 1 mM EDTA [pH 8.0], 0.1% Triton X-100, 0.1% Tween 20, 20 μM phenylmethylsulfonyl fluoride) and sonicated (Vibra Cell VCX500; Sonics and Materials, Inc., Newton, CT) on an ice bed. After sonication, recombinant tag-free Vv-PyrH was purified by using a chitin column and a 50 mM 1,4-dithiothreitol solution in accordance with the manufacturer's protocol, and the purity of the recombinant Vv-PyrH was confirmed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The UMP kinase activity of recombinant PyrH was determined as described elsewhere (2, 3). The reaction mixture contained 50 mM Tris-Cl (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 0.5 mM GTP, and 2 U each of pyruvate kinase, lactate dehydrogenase (LDH), and NDP kinase. Also, 100 nM recombinant Vv-PyrH and 1 mM UMP were sequentially added to the mixture. The decrease in the absorbance at 334 nm was determined. One unit of PyrH corresponds to 1 μmol of UDP formation per 1 min. In order to correct for secondary reactions, the absorbance at 334 nm in the absence of UMP was measured.

Cytotoxicity assay. The CytoTox nonradioactive cytotoxicity assay kit (Promega, Madison, WI) was used to measure the release of cytosolic LDH from dead cells. HeLa cells were seeded in 24-well culture plates at a concentration of 10^5 cells/ml and cultured at 37°C and 5% CO_2. After 24 h, the cells were washed twice with 1 ml of prewarmed serum-free Dulbecco modified Eagle medium.

FIG. 1. Alignment with the amino acid sequences of bacterial PyrH from *V. vulnificus* (Vv), *E. coli* (Ec), *S. enterica* serovar Typhimurium (St), *B. subtilis* (Bs), *L. monocytogenes* (Lm), and *P. furiosus* (Pf). Identical and conserved sequences are in boldface and underlined, respectively. The site-directed mutagenesis (R62H and D77N) was introduced into the sequence indicated by asterisks.

Overnight-cultured *V. vulnificus* cells were inoculated and cultured in fresh 2.5% NaCl HI medium with agitation at 200 rpm for 5 h. The logarithmic growth cultures were harvested by centrifugation, washed three times with phosphate-buffered saline (PBS), and resuspended in PBS to 10^9 CFU/ml. HeLa cells were infected at the multiplicity of infection (MOI) 100. After 120 min of culture, the supernatants were harvested and centrifuged at 13,000 rpm for 5 min at 4°C. A 50-μl portion of the supernatant was transferred to a 96-well plate and mixed with same volume of reconstituted LDH substrate mixture. After 30 min of incubation at room temperature in the dark, a 50-μl portion of stop solution was added to each well, and the absorbance at 490 nm was measured.

LD_{50} determination. The 50% lethal doses (LD_{50}s) of the organism were determined with normal and iron-overloaded mice. For iron-overload experiment, 8-week-old specific-pathogen-free female CD-1 mice were injected intraperitoneally with 900 μg of ferric ammonium citrate (filter sterilized, 100 μg of elemental iron) in PBS for 30 min before bacterial challenge. *V. vulnificus* strains were cultured in 2.5% NaCl HI broth overnight at 37°C with agitation at 200 rpm. Subsequently, 1 ml of the overnight culture was inoculated into 100 ml of fresh 2.5% NaCl HI broth. The cultures were grown at 37°C and 200 rpm for 4.5 h, after which the cells were harvested by centrifugation and washed three times with PBS (pH 7.2). The cell pellet was resuspended and diluted with PBS. Groups of five mice were challenged by intraperitoneal injection of 10-fold serial dilutions of test strains. Deaths were observed for 48 h. The LD_{50} was calculated by the Reed and Muench method (29).

Growth of the bacteria in 2.5% NaCl HI broth, HeLa cell lysate, human ascites, and normal human serum. Growth of the mutant under in vivo-like conditions was examined. *V. vulnificus* cells were cultured and prepared as in the LD_{50} determination described above. The logarithmic-phase bacterial cells were inoculated into 2.5% NaCl HI broth, 50% HeLa cell lysate in PBS, 100% human ascites, and 50% heat inactivated human serum in PBS at concentrations of ca 10^7 CFU/ml and incubated for 6 h at 37°C with agitation. Viable *V. vulnificus* cells were counted on 2.5% NaCl HI agar plates at appropriate time intervals. Growth was not observed in PBS in the absence of HeLa cell lysates or human serum.

Growth of the bacteria in iron-overloaded mice. Growth of the mutant during the infectious process was determined by using iron-overloaded mice. *V. vulnificus* cells were cultured and prepared as described above. The iron-overloaded mice were established as described above. The mice were injected with 10^3 CFU *V. vulnificus* strains, and blood was collected from the mice by heart puncture at various time intervals. Viable bacterial cells were counted on 2.5% NaCl HI agar plates.

Statistical analysis. Results are expressed as means ± the standard errors of the mean (SEM). Statistical comparisons were performed by using Student *t* test to determine the difference between two groups. A P value of <0.05 was considered significant.

RESULTS

Construction of a site-directed *pyrH* mutant strain. To analyze the essential role of PyrH in the in vivo survival and growth of *V. vulnificus*, we introduced point mutations on the critical amino acid residues of the protein. Recently, Briozzo et al. determined the crystal structure of UMP kinase (PyrH) from *E. coli* (2). They showed that the UMP kinase recognizes the UMP substrate through the simultaneous recognition of its base, sugar, and phosphate moieties: the side chain oxygen of Asp77 makes hydrogen bond with 2′OH of ribose and the terminal nitrogen of Arg62 interact with terminal oxygen of alpha-phosphate. *V. vulnificus* PyrH showed 85.5, 85, 51, 48, and 29% amino acid sequence identity with the *E. coli*, *Salmonella enterica* serovar Typhimurium, *Bacillus subtilis*, *L. monocytogenes*, and *P. furiosus* UMP kinases, respectively (2, 9, 18, 24, 30). The two critical substrate-binding residues were con-

TABLE 2. UMP kinase activity of recombinant proteins[a]

Protein	Oligonucleotide sequence (5′–3′)[b]	UMP kinase activity (U/μg)[c]:	
		Without 1 mM UTP	With 1 mM UTP
Wild type	GGTAACTTGTTCCGTGGTGCTGGTCTAGC CGTGTTGTGGGTGACCACATGGGTATG	12.38	6.13
R62H	GGTAACTTGTTCCATGGTGCTGGTCTAGC CGTGTTGTGGGTGACCACATGGGTATG	0.20	ND
D77N	GGTAACTTGTTCCGTGGTGCTGGTCTAGC CGTGTTGTGGGTAACCACATGGGTATG	0.30	ND
R62H/D77N	GGTAACTTGTTCCATGGTGCTGGTCTAGC CGTGTTGTGGGTAACCACATGGGTATG	0.14	ND

[a] The recombinant proteins were purified and the activity of each protein was determined at pH 7.4 using 1 mM UMP as a substrate.
[b] Oligonucleotides were confirmed by sequence analysis. Codon alterations from CGT→CAT (R62N) and GAC→AAC (D77N) are indicated by underlining.
[c] ND, not detectable.

served in all aligned PyrH sequences (FIG. 1). The two UMP binding amino acids were point mutated sequentially. The Arg62 was changed to His and the Asp77 to Asn. At each step, we confirmed the point mutation by restriction enzyme digestion and DNA sequencing analyses.

In order to verify the effect of amino acid modification on the enzymatic activity of PyrH, we manufactured each recombinant protein by using an intein-fusion protein expression system and determined the specific enzymatic activity of the purified mutant proteins. The specific activity of the wild-type PyrH proteins was 12.38 U/μg, and the enzymatic activity was decreased to 6.13 U/μg in the presence of 1 mM UTP as a competitive inhibitor. Compared to wild-type protein, the specific activities of the modified proteins were 1.62, 2.42, and 1.13% for R62H, D77N, and R62H/D77N, respectively (Table 2). This result suggests that the introduction of point mutations at the essential amino acid residues induces a complete inhibition of PyrH activity.

The site-directed mutated gene was then cloned into the suicide vector pDM4, and the resulting plasmids were transferred into the *V. vulnificus* by conjugation. The R62H/D77N site-directed mutation on the *V. vulnificus* chromosome was determined by PCR amplification after NcoI digestion and DNA sequencing analysis (Table 2).

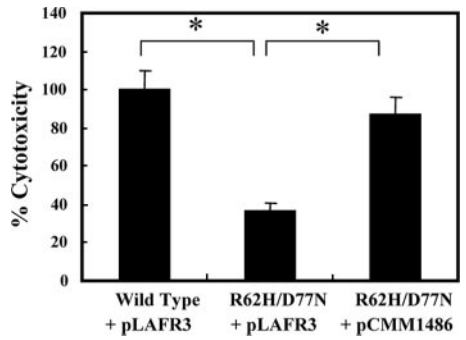

FIG. 2. Effect of the site-directed mutation of *V. vulnificus pyrH* on the cytotoxicity to HeLa cells and its complementation by a wild-type *pyrH* allele. Log-phase bacterial cells were incubated with HeLa cells at an MOI 100 for 120 min. The cytotoxic defect in the R62H/D77N was almost completely complemented by a plasmid carrying a wild-type allele. Values represent the mean percent cytotoxicity ± the SEM. The same experiment was repeated three times with similar results. *, $P < 0.05$.

Effect of R62H/D77N mutation on the cytotoxicity and lethality of *V. vulnificus*. In a previous study, we showed that a *pyrH* insertional mutation resulted in a near abolishment of cytotoxicity and a 14-fold increase in LD_{50} in normal mice (17). To evaluate the effect of the R62H/D77N mutation on *V. vulnificus*, we tested the cytotoxicity and LD_{50} of the mutant. When incubated with HeLa cells at an MOI of 100 for 120 min, the R62H/D77N strain showed significantly decreased LDH release (Fig. 2, $P < 0.005$). The decreased cytotoxicity was fully restored by an in *trans* complementation ($P < 0.05$). In order to rule out the possibility that the antibiotic added to the culture might have retarded the bacterial growth in the complementation group, we compared the complemented strain with the wild-type or the R62H/D77N strain harboring empty pLAFR3 vector. The three test groups were assayed under same culture conditions. We also tested the mouse-killing effect of the strains by measuring LD_{50} against normal and iron-overloaded CD-1 mice. Surprisingly, the intraperitoneal LD_{50} of the mutant increased 26- and 238,000-fold in normal and iron-overloaded mice, respectively (Table 3). This result suggests that R62H/D77N strain has sufficiently attenuated virulence comparable to that of the *pyrH* insertional mutant and that PyrH enzyme activity has an important role in exerting full-blown virulence in vivo.

Growth of the R62H/D77N in human ascites, HeLa cell lysates, and human serum. According to the our previous report (17), the *pyrH* insertional mutant showed a profound growth defect in HeLa cell lysates compared to the wild-type

TABLE 3. Effect of site-directed mutation of *pyrH* on the lethality of *V. vulnificus* in mice

Mouse group	Fold increase in LD_{50}	Intraperitoneal LD_{50} (CFU/mouse)	
		Wild type	R62H/D77N
Normal mice	26	2.1×10^6	5.5×10^7
Iron-overloaded mice	238,000	2.1	5×10^5

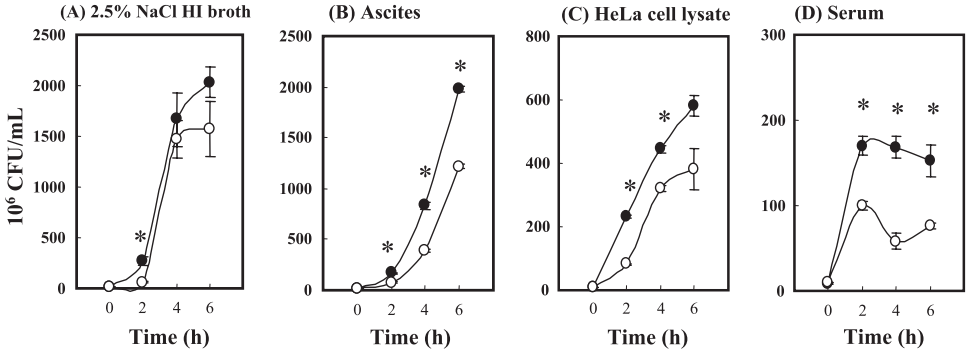

FIG. 3. Growth of the R62H/D77N in 2.5% NaCl HI, human ascites, HeLa cell lysates, and human serum. The logarithmic-phase culture of the R62H/D77N and isogenic wild-type strain were inoculated into 2.5% NaCl HI (A), 100% human ascites (B), 50% HeLa cell lysate in PBS (C), or 50% human serum in PBS (D) and cultured for 6 h at 37°C. Viable cells were counted every 2 h on 2.5% NaCl HI agar plates. All values represent the mean ± the SEM of at least three independent experiments. *, $P < 0.05$.

strain. That result indicates that the *pyrH* mutant should have defects in utilizing host-derived factors for their growth. To test whether the R62H/D77N mutant also had a growth defect under in vivo-like culture conditions, the growth of the mutant in 2.5% NaCl HI broth, 100% human ascites, 50% HeLa cell lysates in PBS, or 50% human serum in PBS was compared to that of the isogenic wild-type strain. The R62H/D77N mutant showed growth retardation even in 2.5% NaCl HI broth until 2 h after incubation. However, the mutant caught up with the growth of the wild type by 4 h. On the other hand, under in vivo-like culture conditions (100% human ascites, 50% HeLa cell lysate, and 50% human serum), the mutant could not recover its growth rate until 4 h. Growth in the 100% ascites was robust, whereas 50% human serum appeared to be the most difficult medium for *V. vulnificus* growth. Viable cell count differences were significant in 100% ascites and 50% serum even at 6 h (Fig. 3). These results suggest that PyrH plays an important role for the growth of *V. vulnificus.* It was also suggested that the necessity of PyrH activity seems to be more noticeable in *V. vulnificus* cells growing in vivo. Notably, the *pyrH* gene was identified as one of the in vivo-expressing genes by in vivo-induced antigen technology (17), and the R62H/D77N mutant showed more growth defects under in vivo-like culture conditions in the present study.

Role of PyrH in the growth of *V. vulnificus* in vivo. To estimate the role of PyrH in the survival of *V. vulnificus* in vivo, we assessed the postinfection recovery of the wild type or the R62H/D77N mutant from infected mice. Iron-overloaded mice were intraperitoneally injected with 10^3 CFU wild-type or R62H/D77N mutant strain, and blood was collected by heart puncture at appropriate time intervals (Fig. 4). Three hours after the injection, the numbers of viable wild type and R62H/D77N mutant organisms in the blood were 2.6×10^3 and 3.1×10^1 CFU/ml, respectively. The viable cell number of the wild-type *V. vulnificus* increased steadily until 9 h up to 5 logs or more cells per ml, and all of the mice died within 10 h after infection. On the other hand, the viable cell count of the R62H/D77N mutant did not significantly change until 9 h after infection, and viable bacteria could not be detected at 24 h after infection (data not shown). These results show that PyrH plays an important role in the growth of *V. vulnificus* in the host milieu.

DISCUSSION

Previously, we have reported that the *V. vulnificus pyrH* gene is an in vivo-expressed genes and that an insertional *pyrH* inactivation resulted in a reduced virulence. In the present study, to characterize the role of *pyrH* during the infectious processes, we first attempted to construct a deletion mutant of *pyrH*, which proved futile after repeated attempts. Instead, we constructed a site-directed *pyrH* mutant strain presumably having a defect in the substrate binding. By using the site-directed mutant we could prove that *pyrH* plays a critical role in the in vivo growth of *V. vulnificus.*

PyrH catalyzes the phosphorylation of UMP to UDP. The resulting UDP is further utilized as an important precursor in the pyrimidine biosynthetic pathway. It has been reported that all of the bacterial genomes reported to date have a conserved *pyrH* gene with no counterpart in eukaryotes (2). Therefore, many efforts have been focused on the biochemical characterization of PyrH in order to develop a new generation of anti-

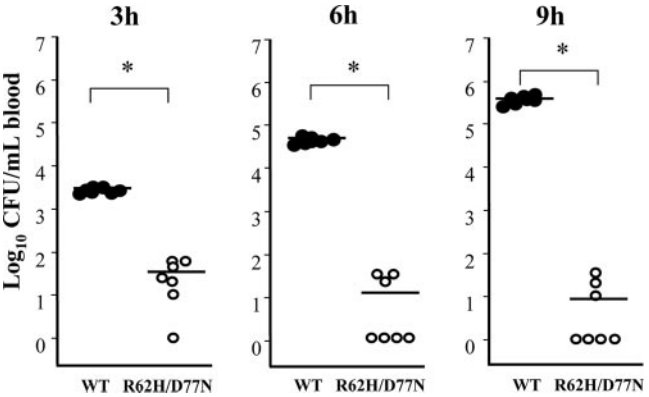

FIG. 4. Recovery of wild type and R62H/D77N mutant from blood after intraperitoneal infection of iron-overloaded mice. BALB/c mice were pretreated with ferric ammonium citrate in PBS for 30 min. The mice were injected with 10^3 CFU *V. vulnificus* strains, and blood was collected from the test mice by heart puncture at appropriate time intervals. Viable cells were counted on 2.5% NaCl HI agar plates at each time point ($n = 7$). The experiment was repeated three times with similar results. Circles indicate each animal, and bars indicate the mean value.

microbial agents (2, 3, 9, 20, 24, 30). Recently, two independent groups determined the molecular structure of PyrH encoded by *E. coli* and *P. furiosus* (2, 24). However, it is hard to find reports directly showing whether the PyrH activity is essential for the in vivo growth and virulence expression of pathogenic bacteria. The present study is the first report using an experimental animal model revealing the essential role of PyrH during the infectious process.

Site-directed mutation at Arg-62, Asp-77, and Arg62+Asp77 resulted in a dramatic decrease of UMP kinase activity (>97% activity loss). These results reconfirmed that these amino acid residues are critical for UMP kinase activity. We then constructed a site-directed *pyrH* mutation on the *V. vulnificus* chromosome by allelic exchange using a suicide plasmid (16, 17, 21). To our knowledge, this is the first report on the construction and evaluation of a site-directed mutation on the *V. vulnificus* chromosome. The R62H/D77N mutation significantly decreased cytotoxicity in HeLa cells. The cytotoxicity defect of the R62H/D77N could be complemented by a wild-type allele, along with its own promoter carried by the IncP plasmid pCMM1486. The decrease in cytotoxicity may have resulted from the growth defect of the R62H/D77N mutant, since growth of the mutant was severely retarded in both HI medium and in vivo-like culture conditions such as 50% HeLa cell lysate. Surprisingly, the lethality of the R62H/D77N mutant was significantly lower than that of the wild-type strain (Table 3): the intraperitoneal LD_{50} for iron-overloaded mice increased 238,000-fold in the R62H/D77N mutant. These data suggest that the introduction of point mutations on the critical *pyrH* gene is sufficient to disturb the UMP kinase activity in vivo. Interestingly, previously tested insertional *pyrH* mutant showed less of an increase (14-fold) in the LD_{50} in normal mice compared to the R62H/D77N mutant (26-fold) (17). When the injected *V. vulnificus* was recruited from blood or livers of moribund mice, almost all (>99.9%) of the bacteria lost inserted plasmid and reverted to wild type (data not shown). Our repeated failure to construct any *pyrH* deletion mutant and this extremely high reversion rate of the insertion mutant strongly support the essentiality of PyrH in the in vivo growth and virulence expression. Changing the two mutated amino acids into wild-type ones should happen very rarely compared to the single-step popping of the suicide plasmid out of the chromosome, although we could not totally rule out the reversion possibility of the R62H/D77N under in vivo situations.

V. vulnificus, an estuarine bacterium, preferentially affects individuals with underlying hepatic disease, heavy alcohol-drinking habits, and other immunocompromised conditions (2, 10, 27, 35, 36). Most underlying diseases are associated with increased iron levels in serum and tissue (1, 4, 10, 19, 38). To reproduce the underlying disease state in a mouse model, we artificially overloaded mice with iron as described earlier (10, 16). The viable bacteria recovery experimental model used here seems to reproduce the real infection process very well. In human infections, a limited number of *V. vulnificus* cells get into circulation and produce progenies utilizing increased free iron in the blood. The establishment of septicemia is the result of active bacterial growth in circulation, for which PyrH activity is essential. Counting viable bacteria in the circulation of iron-overloaded mice would provide more information associated with the host and parasite factors playing important roles in the establishment *V. vulnificus* infection. On the other hand, the LD_{50} determination shows the result of the infection: death or survival. When the iron-overloaded mice were injected with wild type or the R62H/D77N mutant, 2.6×10^3 or 3.1×10^1 of viable wild-type and R62H/D77N mutant *V. vulnificus* organisms, respectively, were recovered from the blood 3 h after infection. This result indicates that both strains could enter into the bloodstream, although the invading efficiencies might be quite different between the strains. At 6 h after infection, the wild type was recovered at levels of 3.6×10^5/ml of blood, whereas the R62H/D77N mutant was recovered at levels approximately 4 log orders lower than the wild type. Live R62H/D77N cells were completely cured from the bloodstream 24 h after infection, whereas the isogenic wild-type strain killed all of the infected mice within 10 h. These results indicate that the R62H/D77N mutant entered into the bloodstream could not replicate well and was eventually removed by the innate immune system such as serum bactericidal activity and mononuclear phagocytes (13, 22, 32, 35). Wild-type *V. vulnificus* seemed to replicate robustly in mice. The estimated doubling time was 72 min in iron-overloaded mice. Conclusively, the R62H/D77N mutant should have a very serious defect in in vivo replication. These results also suggest that the R62H/D77N mutation can be applied to the construction of an in vivo replication controllable live attenuated vaccine strain. *V. vulnificus* capsule is very important for invasiveness and resistance to serum bactericidal activity and phagocytosis (6, 10, 28, 32). UDP serves as a very important precursor for the biosynthesis of capsular polysaccharides (26, 37). A defect in UMP kinase activity should result in a derangement of capsule synthesis. Colonies of the R62H/D77N and insertional mutants on 2.5% NaCl HI agar plates show slightly more translucent morphologies when left at room temperature for more than 48 h (data not shown). However, this possibility should be quantitatively proved by further studies. It has been suggested that PyrH can sense the internal pyrimidine nucleotide pool and regulates the synthase operon *carAB* of carbamoylphosphate that is required for the biosynthesis of arginine and pyrimidines (15). The resulting product, pyrimidine, will act as precursors of the RNA, DNA, and phospholipids. In the case of rapidly growing *V. vulnificus* in vivo, sufficient pools of such precursors are a prerequisite for their multiplication. Therefore, it is quite reasonable that the expression of PyrH should be preferentially upregulated for successful replication during the *V. vulnificus* infection as we predicted in our previous study (17).

In terms of attenuated bacterial vaccine development, the vaccine strain should fulfill two requirements: efficacy and safety (5, 14). First, the vaccine strain should be sufficiently invasive to induce primary and memory immune responses, which finally can induce protective immunity. Second, the strain should possess the features to control in vivo replication of the vaccine strain. The R62H/D77N mutant strain seems to fulfill both requirements. In conclusion, these results suggest that PyrH, essential survival factor of *V. vulnificus*, should serve an attractive new target for the development of antibacterial drug and replication controllable live attenuated vaccines.

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REFERENCES

1. **Brennt, C. E., A. C. Wright, S. K. Dutta, and J. G. Morris, Jr.** 1991. Growth of *Vibrio vulnificus* serum from alcoholics: association with high transferring iron saturation. J. Infect. Dis. **164:**1030–1032.
2. **Briozzo, P., C. Evrin, P. Meyer, L. Assairi, N. Joly, O. Barzu, and A. M. Gilles.** 2005. Structure of *Escherichia coli* UMP kinase differs from that of other nucleoside monophosphate kinases and sheds new light on enzyme regulation. J. Biol. Chem. **280:**25533–25540.
3. **Bucurenci. N., L. Serina, C. Zaharia, S. Landais, A. Danchin, and O. Barzu.** 1998. Mutational analysis of UMP kinase from *Escherichia coli*. J. Bacteriol. **180:**473–477.
4. **Bullen, J. J., P. B. Spalding, C. G. Ward, and J. M. Gutteridge.** 1991. Hemochromatosis, iron and septicemia caused by *Vibrio vulnificus*. Arch. Intern. Med. **151:**1606–1609.
5. **Curtiss, R., III.** 2002. Bacterial infectious disease control by vaccine development. J. Clin. Investig. **110:**1061–1066.
6. **Devi, S. J., U. Hayat, J. L. Powell, and J. G. Morris, Jr.** 1996. Preclinical immunoprophylactic and immunotherapeutic efficacy of antisera to capsular polysaccharide-tetanus toxoid conjugate vaccines of *Vibrio vulnificus*. Infect. Immun. **64:**2220–2224.
7. **Ditta, G., D. C. Stanfield, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
8. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
9. **Gagyi, C., N. Bucurenci, O. Sirbu, G. Labesse, M. Ionescu, A. Ofiteru, L. Assairi, S. Landais, A. Danchin, O. Barzu, and A. M. Gilles.** 2003. UMP kinase from the gram-positive bacterium *Bacillus subtilis* is strongly dependent on GTP for optimal activity. Eur. J. Biochem. **270:**3196–3204.
10. **Gulig, P. A., K. L. Bourdage, and A. M. Starks.** 2005. Molecular Pathogenesis of *Vibrio vulnificus*. J. Microbiol. **43:**118–131.
11. **Heithoff, D. M., C. P. Conner, P. C. Hanna, S. M. Julio, U. Hentschel, and M. J. Mahan.** 1997. Bacterial infection as assessed by in vivo gene expression. Proc. Natl. Acad. Sci. USA **94:**934–939.
12. **Heithoff, D. M., C. P. Conner, and M. J. Mahan.** 1997. Dissecting the biology of a pathogen during infection. Trends Microbiol. **5:**509–513.
13. **Hor, L. I., T. T. Chang, and S. T. Wang.** 1999. Survival of *Vibrio vulnificus* in whole blood from patients with chronic liver diseases: association with phagocytosis by neutrophils and serum ferritin levels. J. Infect. Dis. **179:**275–278.
14. **Kang, H. Y., J. Srinivasan, and R. Curtiss III.** 2002. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica* serovar Typhimurium vaccine. Infect. Immun. **70:**1739–1749.
15. **Kholti, A., D. Charlier, D. Gigot, N. Huysveld, M. Roovers, and N. Glansdorff.** 1998. *pyrH*-encoded UMP-kinase directly participates in pyrimidine-specific modulation of promoter activity in *Escherichia coli*. J. Mol. Biol. **280:**571–582.
16. **Kim, S. Y., S. E. Lee, Y. R. Kim, C. M. Kim, P. Y. Ryu, H. E. Choy, S. S. Chung, and J. H. Rhee.** 2003. Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. Mol. Microbiol. **48:**1647–1664.
17. **Kim, Y. R., S. E. Lee, C. M. Kim, S. Y. Kim, E. K. Shin, D. H. Shin, S. S. Chung, H. E. Choy, A. Progulske-Fox, J. D. Hillman, M. Handfield, and J. H. Rhee.** 2003. Characterization and pathogenic significance of *Vibrio vulnificus* antigens preferentially expressed in septicemic patients. Infect. Immun. **71:**5461–5471.
18. **Klarsfeld, A. D., P. L. Goossens, and P. Cossart.** 1994. Five *Listeria monocytogenes* genes preferentially expressed in infected mammalian cells: *plcA*, *purH*, *purD*, *pyrE*, and an arginine ABC transporter gene, *arpJ*. Mol. Microbiol. **13:**585–597.
19. **Kraffert, C. A., and D. J. Hogan.** 1992. *Vibrio vulnificus* infection and iron overload. J. Am. Acad. Dermatol. **26:**140.
20. **Labesse, G., N. Bucurenci, D. Douguet, H. Sakamoto, S. Landais, C. Gagyi, A. M. Gilles, and O. Barzu.** 2002. Comparative modeling and immunochemical reactivity of *Escherichia coli* UMP kinase. Biochem. Biophys. Res. Commun. **294:**173–179.
21. **Lee, S. E., S. H. Shin, S. Y. Kim, Y. R. Kim, D. H. Shin, S. S. Chung, Z. H. Lee, J. Y. Lee, K. C. Jeong, S. H. Choi, and J. H. Rhee.** 2000. *Vibrio vulnificus* has the transmembrane transcription activator ToxRS stimulating the expression of the hemolysin gene *vvhA*. J. Bacteriol. **182:**3405–3415.
22. **Linkous, D. A., and J. D. Oliver.** 1999. Pathogenesis of *Vibrio vulnificus*. FEMS Microbiol. Lett. **174:**207–214.
23. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686–688.
24. **Marco-Marin, C., F. Gil-Ortiz, and V. Rubio.** 2005. The crystal structure of *Pyrococcus furiosus* UMP kinase provides insight into catalysis and regulation in microbial pyrimidine nucleotide biosynthesis. J. Mol. Biol. **352:**438–454.
25. **Milton, D. L., R. O'Toole, P. Horstedt, and H. Wolf-Watz.** 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. J. Bacteriol. **178:**1310–1319.
26. **Neuhard, J., and R. A. Kelln.** 1996. Biosynthesis and conversion of pyrimidines, p. 580–599. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, DC.
27. **Park, S. D., H. S. Shon, and N. J. Joh.** 1991. *Vibrio vulnificus* septicemia in Korea: clinical and epidemiologic findings in seventy patients. J. Am. Acad. Dermatol. **24:**397–403.
28. **Reddy, G. P., U. Hayat, C. Abeygunawardana, C. Fox, A. C. Wright, D. R. Maneval, Jr., C. A. Bush, and J. G. Morris, Jr.** 1992. Purification and determination of the structure of capsular polysaccharide of *Vibrio vulnificus* MO6-24. J. Bacteriol. **174:**2620–2630.
29. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating the fifty percent end points. Am. J. Hyg. **27:**493–497.
30. **Sakamoto, H., S. Landais, C. Evrin, C. Laurent-Winter, O. Barzu, and R. A. Kelln.** 2004. Structure-function relationships of UMP kinases from *pyrH* mutants of gram-negative bacteria. Microbiology **150:**2153–2159.
31. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32. **Shinoda, S., M. Kobayashi, H. Yamada, S. Yoshida, M. Ogawa, and Y. Mizuguchi.** 1987. Inhibitory effect of capsular antigen of *Vibrio vulnificus* on bactericidal activity of human serum. Microbiol. Immunol. **31:**393–401.
33. **Simon, R., R. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Biotechnology **1:**784–791.
34. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. glyxinea. J. Bacteriol. **169:**5789–5794.
35. **Strom, M. S., and R. N. Paranjpye.** 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microbes Infect. **2:**177–188.
36. **Tacket, C. O., F. Brenner, and P. A. Blake.** 1984. Clinical features and an epidemiological study of *Vibrio vulnificus* infections. J. Infect. Dis. **149:**558–561.
37. **Ventura, C. L., R. T. Cartee, W. T. Forsee, and J. Yother.** 2006. Control of capsular polysaccharide chain length by UDP-sugar substrate concentrations in *Streptococcus pneumoniae*. Mol. Microbiol. **61:**723–733.
38. **Wright, A. C., L. M. Simpson, and J. D. Oliver.** 1981. Role of iron in the pathogenesis of *Vibrio vulnificus* infections. Infect. Immun. **34:**503–507.

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