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Nonribosomal Peptide Synthase is Responsible for the Biosynthesis of Siderophore in *Vibrio vulnificus* MO6-24/O

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Vibrio vulnificus **produces siderophores, low-molecular-weight iron-chelating compounds, to obtain iron under conditions of iron deprivation. To identify genes associated with the biosynthesis of siderophore in *V. vulnificus* MO6-24/O, we screened clones with mini-Tn*5* random insertions for those showing decreased production of siderophore. Among 6,000 clones screened, nine such clones were selected. These clones contain the transposon inserted in VV2_0830 (GenBank accession number) that is a homolog of a nonribosomal peptide synthase (NRPS). There is an another NRPS module, VV2_0831, 49-bp upstream to VV2_0830. We named these two genes *vvs* (*Vibrio vulnificus* siderophore synthase) *A* and *B*, respectively. Mutation of either *vvsA* or *vvsB* showed a decreased production of siderophore. The expression of an NRPS-*lux* fusion was negatively modulated by the presence of iron, and the regulation was dependent on Fur (ferric uptake regulator). However, the expression of the NRPS genes was still not fully derepressed in the iron-rich condition, even in *fur*-null mutant cells, suggesting that some other unknown factors are involved in the regulation of the genes. We also demonstrated that the NRPS genes are important for virulence of the pathogen in a mice model.**

Keywords: *Vibrio vulnificus*, siderophore, nonribosomal peptide synthase

Virtually all organisms require iron for growth, but iron availability is limited in most aerobic environments. Iron is essential for bacterial growth, and the ability to acquire iron from the host is a prerequisite for pathogenicity [35]. The importance of iron for microorganisms has long been recognized, and the availability of host iron has been proposed as a contributing factor in a number of experimental bacterial infections [34] as well as an important factor in affecting the expression of genes with various functions [20]. Iron within the mammalian host is bound to various proteins and is, therefore, not readily available for bacterial acquisition. For example, the bactericidal or bacteriostatic properties of serum have been attributed to the sequestering of iron by transferring [36].

Many microorganisms have developed efficient means to obtain iron and, under conditions of iron deprivation, to produce low-molecular-weight iron-chelating compounds, called siderophores [16]. These compounds are synthesized and secreted into the environment, where they bind ferric iron with high affinity [37]. In Gram-negative bacteria, outer membrane receptors, including FepA in *Escherichia coli* [23] and ViuA in *Vibrio cholerae* [4], bind the ferrisiderophore complex. Uptake of the ferrisiderophore into the cell is dependent upon TonB and specific periplasmic and inner membrane proteins [15]. Through the membrane-bound transport system, these compounds are transported across the lipid-protein boundary. Iron is then removed from the chelator complex for use by the cell. Vibriobactin, a known siderophore produced by *V. cholerae*, is a catechol [12]. The prototype catechol, enterobactin, which is produced by *E. coli*, is a cyclic trimer of 2,3-dihydroxybenzoyl-L-serine [25]. Vibriobactin also contains three 2,3-dihydroxybenzoyl residues, but they are linked to a backbone of norspermidine, a compound rarely encountered in bacterial species [12]. Two of the 2,3-dihydroxybenzoyl moieties are linked to the backbone *via* L-threonine residues, whereas the third is directly linked to the norspermidine [37].

V. vulnificus is a Gram-negative halophilic marine bacterium that has been associated with primary septicemia and serious wound infections [14, 21], and a new siderophore, named vulnibactin, has been isolated from low-iron cultures of this pathogen. The structure was established as *N*-[3-(2,3-dihydroxybenzamido) propyl]-1,3-bis[2-(2-hydroxyphenyl)-trans-5-methyl-2-oxazoline-4-carboxamido] propane.

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Vulnibactin is shown to contain one residue of 2,3-dihydroxybenzoic acid and two residues of salicylic acid, both of which are involved in the formation of oxazoline rings with L-threonine, which is bound to a norspermidine backbone [26]. However, the mechanism of the biosynthetic pathway of siderophore in this bacterium has remained to be elucidated. In this study, we identified genes responsible for the biosynthesis of a siderophore in the pathogen.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth with aeration at 37°C. *V. vulnificus* strains were grown in either LB or TCBS agar (Difco) at 28°C. The AB medium was used as a minimal medium and was prepared as follows; 0.3 M NaCl, 50 mM $MgSO_4 \cdot 7H_2O$, and 0.2% casamino acids were mixed in double-distilled water, then the pH was adjusted to 7.5 with KOH and the medium autoclaved. Before using, the minimal medium was mixed with potassium phosphate buffer (pH 7.0), 1× vitamin solution (v/v), 1% glycerol (v/v), and 1 mM L-arginine supplemented. Antibiotics at the following concentration were used; 100 μg/ml kanamycin and 2 μg/ml tetracycline for *V. vulnificus*; 10 μg/ml tetracycline, 50 μg/ml ampicillin, and 25 μg/ml kanamycin for *E. coli*. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a concentration of 40 μg/ml.

Growth in Iron-limiting and Iron-rich Conditions

For the iron-limiting condition, we used AB medium supplemented with the iron chelator, 2 μM ethylenediamine di *ortho*-hydroxyphenylacetic acid (EDDA) [22]. AB was supplemented with 50 μM ferric chloride for the iron-rich condition.

Construction of the Cosmid Genomic Library of *V. vulnificus* MO6-24/0

Total genomic DNA was prepared from *V. vulnificus* as described previously [19]. Briefly, *V. vulnificus* was grown overnight in LB medium, and cells at the stationary phase were harvested by centrifugation. The cells were resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and lysated with 5% sarkosyl and protease K (5 mg/ml) by incubation at 37°C until complete lysis of cells occurred. The lysate was extracted three times with phenol saturated with 3% NaCl and subsequently extracted with chloroform:isoamyl alcohol (24:1) and diethyl ether. Then, total DNA was precipitated by 95% ethanol and the precipitate was washed with 70% ethanol. Finally, total DNA was resuspended in TE. The cosmid pCP13/B was used for construction of the genomic library of *V. vulnificus*, as described previously [29].

Chrome Azurol S (CAS) Assay

Medium for the siderophore assay was prepared as described previously [6]. The CAS agar was prepared as follows; for 1 l of CAS agar, the mixture of 6.0 g NaOH, 30.24 g PIPES, 1× MM9 and 1.5% Bacto-agar was autoclaved and cooled to 50°C. Subsequently, 30 ml of deferrated casamino acids, 0.2% glucose (v/v), 1 mM $MgCl_2$, 100 μM $CaCl_2$, and 100 ml of CAS-HDTMA (hexadecyltrimethylammonium bromide) solution were added, and mixed gently. For liquid assay, CAS liquid solution was employed. Briefly, 0.5 ml of CAS solution (composed of 0.015 mM Fe solution, 0.15 mM CAS stock solution, piperazine buffer, and HDTMA) was added to 0.5 ml of cell-free culture supernatant, and they were mixed. Then, 10 μl of shuttle solution (0.2 M 5-

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotypes and characteristics	Source or reference
Strains		
Escherichia coli		
DH5α	SupE44 ΔlacU169(801acZΔM15) hsd 17 secA1 gyrA9 *Thi-1 relA1,* Nal^r	[28]
S17-1	*recA, thi, pro, hsdR⁻ M⁻* [RP4-2Tc::Mu::Km^r::Tn*7*], Tp^r Str^r	[30]
S17-1 *λpir*	*recA, thi, pro, hsdR⁻ M⁻* [RP4-2Tc::Mu::Km^r::Tn*7*] *(λpir),* Tp^r Str^r	[8]
Vibrio vulnificus		
MO6-24/O	Clinical isolate	[27]
VNH200	Derivative of MO6-24/O with a mini-Tn*5lacZ1* insertion in *vvsB*	This study
VNH201	Derivative of MO6-24/O with a deletion in *vvsA*	This study
VNH202	Derivative of MO6-24/O with a deletion in both *vvsA* and *vvsB*	This study
HLM101	Derivative of MO6-24/O with a deletion in *fur*	[16]
Plasmids		
pDM4	A suicide vector for allelic exchanges, R6K, Cm^r	[27]
pHK0011	Derivative of pRK415 containing a promoterless *luxAB*, Tc^r	[5]
pCP13/B	$mob^+tra^-Tc^r$, low-copy-number, RK2 broad-host-range vector	[9]
pBluescript SK (−)	Cloning vector, Ap^r	Our collection
pGemT Easy vector	Cloning vector, Ap^r	Promega
pCP3, pCP4, pCP5	Clones of the *V. vulnificus* cosmid genomic library	This study
pHKN	Derivative of pHK0011 with the promoter region of *vvsA* transcriptionally fused to *luxAB*	This study

sulfosalicylic acid) was added, and the mixture was left for a few minutes. If siderophores were present, the color of the solution turns blue, indicating that iron was removed from the dye complex. Absorbance A_{630} was measured for the loss of siderophore, using the minimal medium as a blank and the minimal medium plus CAS assay solution and shuttle solution as a reference. The sample should have a reading lower than the references. Siderophore units were defined as [(OD_{630} of reference–OD_{630} of sample)/OD_{630} of reference]×100=% siderophore units.

Transposon Mutagenesis and Selection of Cosmid Clones Complementing the Defect of the Siderophore Production

V. vulnificus genome was randomly inserted by miniTn*5* as described previously [9, 13]. We obtained a total of 6,000 colonies, and each of the mutagenized clones was tested on CAS agar plates for the loss of siderophore production. For the complementation test, we introduced cosmid libraries of *V. vulnificus* MO6-24/O genomic DNA into *E. coli* strain S17-1, which was subsequently conjugated with a derivative of *V. vulnificus* MO6-24/O, which had a mutation in a nonribosomal peptide synthase (NRPS). The resulting exconjugants were screened for the recovery of siderophore production.

Construction of a Reporter Fusion to the Promoter of NRPS Genes

A 276-bp fragment containing the promoter region of *vvsA* was amplified by PCR using primers, p831-F (5'-GGTACCAATTTGCTGAGCGTAG-3') and p831-B (5'-GGATCCCCACCCAAATTTAC-3'), and *V. vulnificus* genomic DNA as a template. Reactions consisted of 30 cycles of 30 sec at 94°C for denaturation, 30 sec at 54°C for annealing, and 30 sec for extension at 72°C, followed by a single cycle at 72°C for 10 min. The PCR product was cloned in the pGemT-Easy vector (Promega, Madison, WI, U.S.A.). A BamHI-KpnI fragment from the pGemT-Easy vector was subcloned into pHK0011 [5], which carries a promotorless *lux*AB.

Luminescence Assay

Luciferase activities were determined with a luminometer (LB9506, Berthold Technologies, Germany) by adding *n*-decyl aldehyde solution (0.006% in 50% ethanol). Overnight cultures were washed twice with fresh AB minimal medium and resuspended in tetracycline (2 μg/ml) containing AB minimal medium to adjust the initial optical density to 0.1. The level of expression was monitored throughout the growth stages at 28°C. The relative light unit was expressed as specific bioluminescence divided by the cell density (optical density at 600 nm [OD_{600}]).

LD_{50} Determination

Cells were grown in LB broth until the stationary phase. Cells were harvested by centrifugation at 11,000 rpm for 5 min at room temperature, washed with phosphate-buffered saline (PBS) (pH 7.4), and resuspended and diluted in PBS. Viable counts were measured by spotting using LB-agar plates. The plates were incubated at 37°C

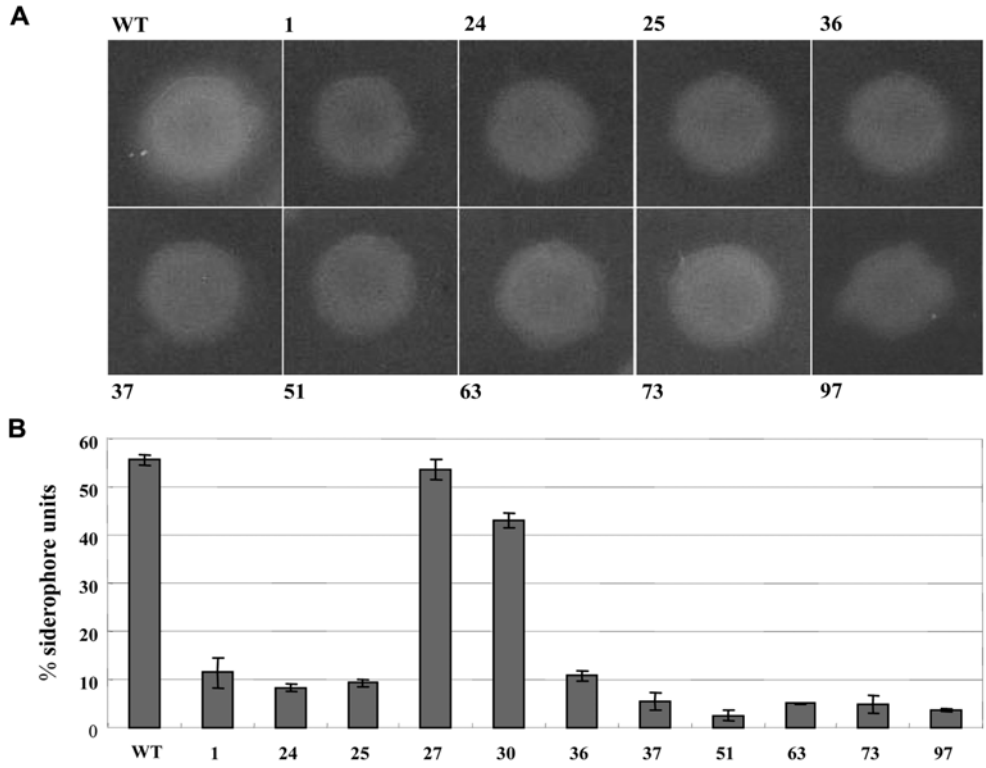


Fig. 1. Siderophore production by *V. vulnificus* cells with mini-Tn*5* insertions.

A. Siderophore production as detected on CAS agar plates. Wild type produces an orange halo around the colony, which denotes the siderophore activity, but mutant cells do not. **B**. Siderophore production in an iron-limiting condition. The ability to produce siderophore is expressed as percentage siderophore units, as quantitatively measured by CAS liquid assay. These results came from a single representative experiment out of experiments performed in triplicate, with error bars showing standard deviations. Each number represents the clone number of mini-Tn*5* insertion clones.

and colonies were counted after 24 h. Four-week-old ICR female mice (Samtako Bio, Korea) were housed under specific-pathogen-free conditions. Ten-fold serial dilutions of suspended cells in 0.1 ml of PBS were hypodermically injected into the back of groups of 3 mice. Control mice were injected with PBS only. The LD_{50} value was calculated as reported previously [18].

Results

Mutation in a Nonribosomal Peptide Synthase Abolishes the Production of Siderophore

To investigate the genes responsible for biosynthesis of a siderophore in *V. vulnificus*, the mini-Tn*5* random insertion mutant library generated on the genome of MO6-24/O was examined. About 6,000 insertional mutant clones were tested on CAS agar plates. Among them, nine mutant cells that were not able to produce the halo around a colony were selected (Fig. 1A) and confirmed by quantitative CAS liquid assay (Fig. 1B). We isolated genomic DNA from each of these clones, and the DNA was digested using several restriction enzymes. We carried out Southern hybridization using the Tn*5* fragment as a probe to determine the restriction fragment with the insertion mutation (data not shown). The result showed that Tn*5* insertions in eight mutants (mutant numbers 1, 24, 25, 36, 37, 51, 63, and 97 in Fig. 1) were located at the same position in chromosome I, and one mutant (mutant number 73) had its mutation mapped in chromosome II. We determined the sequences of DNA from these insertion mutants and found that the insertions in the mutant 73 were mapped in a homolog of nonribosomal peptide synthase (NRPS; GenBank Accession No. VV2_0830). The rest of the mutants are now under investigation. This gene had 67% similarity and 52% identity with *vibF*, which encodes a function responsible for biosynthesis of siderophore in *Vibrio cholerae*. We constructed an internal inframe deletion in VV2_0830 by an allelic exchange. The VV2_0830-null mutant cells (VNH200, 201, 203) showed just a basal level of siderophore activity compared with wild-type cells, as determined by the CAS liquid assay (Fig. 2).

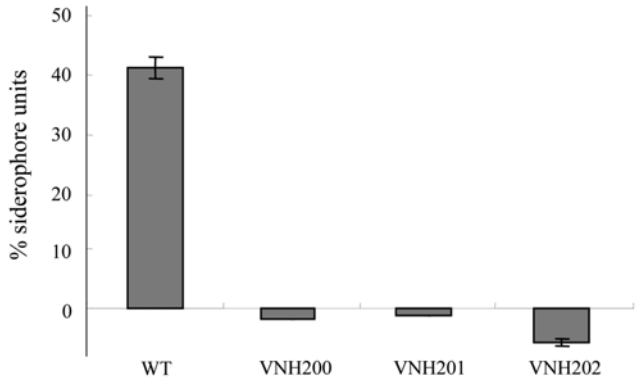


Fig. 2. Quantitative analysis of siderophore activity as measured by CAS liquid assay.
VNH200 carries a mutation in *vvsB*, VNH201 in *vvsA*, and VNH202 carries mutations in both *vvsA* and *vvsB*.

Another NRPS Gene, VV2_0831, Adjacent to VV2_0830 is also Involved in Siderophore Production

The DNA nucleotide sequence of the region upstream to VV2_0830 indicated the existence of a gene, VV2_0831, which putatively encoded another NRPS that is a homolog of *vibF*. To examine whether VV2_0831 is also involved in siderophore production, we constructed an inframe deletion mutation in the gene. As shown in Fig. 2, *V. vulnificus* MO6-24/O cells with a mutation either in VV2_0830 (VNH200) or in 20831 (VNH201) as well as cells with mutation in both of the genes (VNH202) completely lost the ability to produce siderophore, suggesting that VV2_0831 is also necessary for the siderophore production. We named the VV2_0831 and VV2_0830 genes to be *vvs* (*Vibrio vulnificus* siderophore synthase) *A* and *B*, respectively.

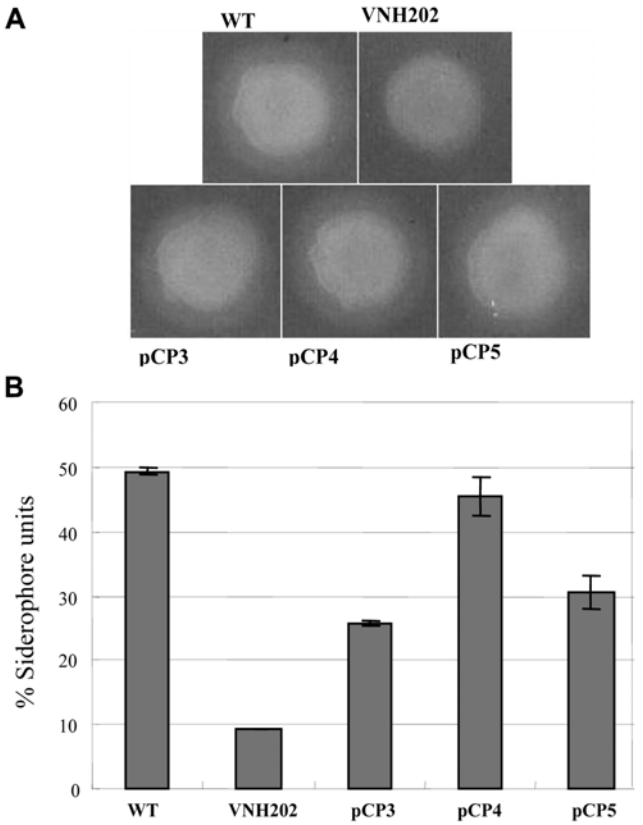


Fig. 3. Complementation of siderophore production by *V. vulnificus* cosmid clones.
A. Siderophore production as detected on CAS agar plates. Mutation in *vvsB* abolished the production of siderophore, and the library clones pCP3, 4, and 5 restored the activity. The orange hallow around a colony indicates the activity of siderophore. **B**. Quantitative analysis of siderophore activity, as measured by CAS liquid assay.

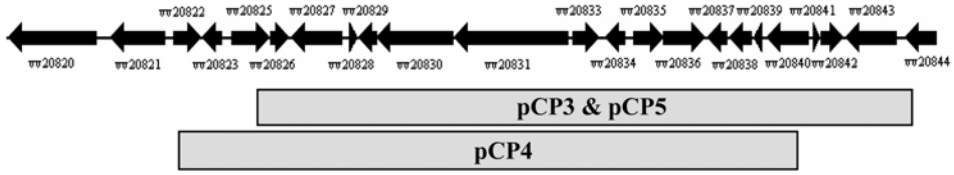
Selection of Cosmid Clones of *V. vulnificus* MO6-24/O that Complements the Defect of Siderophore Production

A genomic library of *V. vulnificus*, constructed in the cosmid pCP13/B, was transferred into the NRPS-deficient strain VNH202, which has double mutations in *vvsA* and *vvsB*, by biparental mating, and siderophore production was examined for on CAS agar plates. Among 3,000 transconjugants obtained, 3 clones restored the ability to form an orange halo around their colonies (Fig. 3A). The cosmid constructions in these clones were named as pCP3, pCP4, and pCP5, respectively. Quantitative assay for the siderophore activity showed that pCP4 restored the siderophore activity up to the level of the wild-type, whereas pCP3 and pCP5 partially restored the activity (Fig. 3B). Restriction profiles of the inserts in these cosmids indicated that they carry a common restriction region (Fig. 4): All three cosmids carried the two *vibF* homologs, *vvsA* and *vvsB* (Fig. 4). In contrast to pCP3 and pCP5, pCP4 carried an additional gene, VV2_0823, that putatively encodes an ABC-type transporter. These results confirmed that the two *vibF* homologs are responsible for the siderophore production in *V. vulnificus*, and suggested that the additional function present in pCP4 is necessary for full production of siderophore.

Iron Affects the Expression of NRPS Genes in *V. vulnificus*

It has been well documented that functions for siderophore production and transport are regulated by Fur, the transcriptional repressor for the genes involved in iron acquisition and iron metabolism in many bacteria [11, 27, 33]. Furthermore, the sequence analysis indicated that there exists a *fur* box in the *vvsA* promoter region (Fig. 5). Therefore, to determine whether the expression of these two *vibF* homologs is also regulated by Fur in *V. vulnificus*, we quantitatively measured the production of siderophore in wild type and in an isogenic *fur* null-mutant by using the liquid CAS assay (Fig. 6). In normal or iron-

A



B

Gene	Size	Product	Features
VV2_0820	3564	Uncharacterized protein contain chitin-binding domain type 3	
VV2_0821	2178	FOG: EAL domain protein	
VV2_0822	1086	Hypothetical protein	
VV2_0823	750	ABC-type amino acid transport/signal transduction systems	
VV2_0825	1491	Methyl-accepting chemotaxis protein	
VV2_0826	729	ABC-type amino acid transport/signal transduction system	
VV2_0827	2043	Predicted phosphatase	
VV2_0828	339	Hypothetical protein	
VV2_0829	696	Phosphopantetheinyl transferase component of siderophore synthetase	
VV2_0830	**3069**	**Nonribosomal peptide synthetase modules**	***vibF***
VV2_0831	**4563**	**Nonribosomal peptide synthetase modules**	***vibF***
VV2_0833	1071	3-Deoxy-7-phosphoheptulonate synthase	
VV2_0834	789	2,3-Dihydroxybenzoate-2,3-dehydrogenase	
VV2_0835	1182	Isochorismate synthase	
VV2_0836	1638	Peptide arylation enzyme	
VV2_0837	816	Siderophore-interacting protein	*viuB*
VV2_0838	888	Aryl carrier domain	*vibB*
VV2_0839	330	Chorismate mutase	
VV2_0840	1629	Peptide arylation enzyme	
VV2_0841	243	Aryl carrier domain	
VV2_0842	888	ABC-type enterochelin transport system, periplasmic component	
VV2_0843	2058	Outer membrane receptor protein	*viuA*
VV2_0844	1284	Nonribosomal peptide synthetase modules	*vibH*

Fig. 4. Genetic organization of NRPS and neighbor genes in *V. vulnificus*.

A. A genetic map of library clones pCP3, 4, and 5. **B**. A list of genes, which are contained in the library clones. BLAST search showed that genes related to the iron acquisition system of *V. vulnificus* are homologous to those of *V. cholerae*.

GTTTCCCCCT GAGCCATATC GGTAACTGAG TTGTAAAAAT AAATAATGTT AATGATAATT ATTATCGTTA

ATGTCGGAAT TATTAAGAAA GAGGAATCGA TATGTCAAGC CGAAATCTTG CTCAAGTCAC CCTATGCTAA

CCAGTAAAAT CAACAAGATG AACTGACATT CTTTCACTAC AAGCCTCGTT GTTTGGCATT TTAATTACTT

TACATGTAAA TGATA*ATGAT TATTATTAAT GTAAATTTGG GTGGCGAAAT GCTATCAAAT TGTGCGCCGA*

vvsA

CAGACGACAG ACCGACAGGC GAAGTGAATG GACTATGTTT GGATTTTAAC ATGAAAGAAATGACGGCAAT

GCAGGCCGCT

Fig. 5. The sequence of the putative promoter region of *vvsA*.
The coding region of *vvsA* is shown in italics, and the putative *fur* box is underlined.

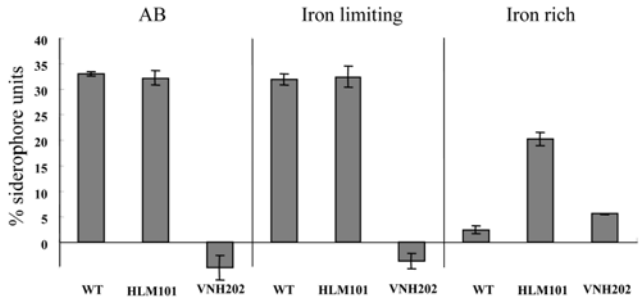


Fig. 6. Siderophore activities of *V. vulnificus* grown in different concentrations of iron.
For the iron-limiting condition, AB medium was supplemented with 2 μM EDDA, and 50 μM ferric chloride was supplemented for the iron-rich condition. These results are from experiments performed in triplicate. Error bars denote standard deviations.

limiting conditions, the *fur*⁻ mutant showed siderophore activity as high as the wild type. However, in iron-rich condition, the wild-type cells did not show a significant level of the siderophore activity. In contrast, the *fur*⁻ mutant still showed the activity, but the level was only half of that shown under iron-limiting condition. These suggested that the synthesis of siderophore is modulated by iron concentration, and that the regulation in *V. vulnificus* is mediated by *fur*. Next, a *vvsA*::*luxAB* transcriptional fusion was constructed, and its expression was quantitatively compared between iron-rich and iron-limiting conditions (Fig. 7). The expression of the *vibF* homolog, *vvsA*, in the iron-limiting condition was 100 times higher than that in the iron-rich condition. These results suggested that the NRPS gene is modulated by iron concentration, and that the regulation is mediated by Fur at the transcriptional level.

NRPS is Important for the Virulence of *V. vulnificus*

To determine whether the expression of the NRPS gene is correlated with the virulence of *V. vulnificus*, we infected 4-week-old mouse with the wild type and a NRPS-null mutant strain. The LD_{50} of the mutant strain VHN202 (NRPS-deficient strain) was 1.03×10^8, whereas the LD_{50} was 5.26×10^5 for the wild type (MO6-24/O), suggesting that the ability to produce siderophore and NRPS are important for virulence in this animal model.

DISCUSSION

Animal pathogens confront a difficulty in obtaining iron because soluble ion is scarce in nature, and furthermore, iron is present not in a free form but in complexes with iron-binding proteins in mammalian hosts. As a consequence, bacteria are equipped with machineries to acquire iron from the environment, and the synthesis of many toxins and virulence determinants is regulated by the intracellular iron concentration of bacterial cells [24]. Similar to other invasive bacterial pathogens, iron-scavenging siderophores and proteins that bind host iron-containing proteins, such as catechol and hydroxamate siderophore, have been identified in *V. vulnificus* [30]. Vulnibactin, one of the catechol-like siderophores produced by *V. vulnificus*, was determined to be structurally a dihydroxybenzoic acid-containing compound [26]. It is now clear that the iron acquisition system and the virulence of *V. vulnificus* are closely related [2, 17, 21, 26].

V. vulnificus produces at least three different types of siderophores [26]. Among them, vulnibactin is the major siderophore in the pathogen, and its chemical structure is

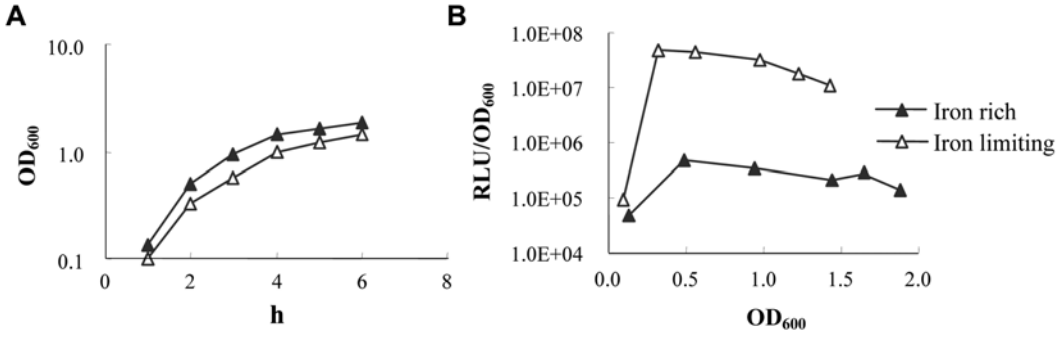


Fig. 7. Expression of the NRPS gene is dependent on iron concentration.
A. Growth curve of cells. **B**. Luciferase activities are expressed as normalized values. For the iron-limiting condition, 2 μM EDDA was added (open symbol), and 50 μM ferric chloride was added for the iron-rich condition (closed symbol). This result is from a single representative experiment out of experiments performed in triplicate. RLU, relative light units.

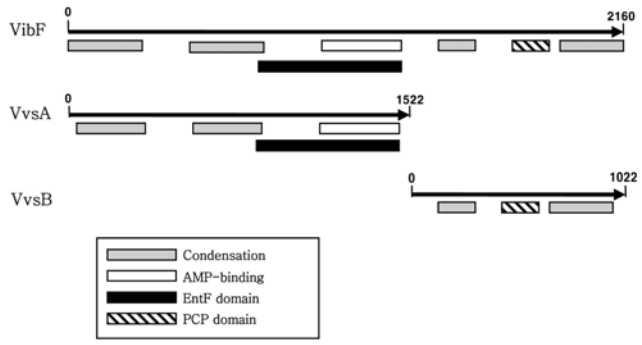


Fig. 8. Comparisons of structures and domains among VibF, VvsA, and VvsB.
The numbers over the map denote the coordination numbers of amino acid residues. The annotation of functional domains was adapted from a earlier review [7].

closely related with that of vibriobactin from *V. cholerae* [12, 26]. In this study, we identified two NRPS homologs, *vvsA* and *vvsB*, which are responsible for the vulnibactin synthesis in *V. vulnificus*. The mutations in *vvsA* and *vvsB*, alone or both, showed only a basal level of siderophore activity (Fig. 2), suggesting that both these two genes are required for the production of vulnibactin. It is of interest to note that the deduced amino acids of these two genes have homologies with the C-terminal and N-terminal of VibF, respectively, which is an NRPS synthase in *V. cholerae* responsible for the production of the siderophore vibriobactin [3, 4] (Fig. 8). It is quite likely that *vvsA* and *vvsB* were evolved from a single gene by splitting, similar to *vibF*. The biochemical basis of the vibriobactin synthesis has been inferred, based upon protein domain homology searches [3, 8]. Considering the strong homology between *vibF* and *vvs* genes and also the strong similarity in chemical structures between vibriobactin and vulnibactin, it is expected that VvsA is involved in AMP-binding and condensation, and VvsB is responsible for phosphopantetheinyl attachment and elongation. It should also be noted that *vvsB* in its length and the organization of functional domains is homologous to *angM*, which is responsible for siderophore production in a fish pathogen, *V. anguillarum* [22] (data not shown).

The expression of *vibF* in *V. cholerae* is negatively regulated by Fur in coordination with iron level. Likewise, we observed that the expression of *vvs* genes was also regulated by iron level, and that the regulation was mediated by Fur.

In iron-limiting conditions, *vvs* genes are fully expressed in both wild-type cells and isogenic *fur*-mutant cells. In iron-rich condition, the expression of *vvs* was fully repressed in the wild type; however, the *vvs* expression level in the *fur*-mutant was higher than that in the wild type, but not fully derepressed. These results suggest that the expression of *vvs* in *V. vulnificus* is negatively regulated by Fur in the presence of iron, and some additional factor(s) is involved in the regulation of siderophore synthesis. Previous study showed that *vibF* is also regulated by *irgB*, which is a positive regulator associated with the regulation of virulence factors *irgA* in *V. cholerae* [32]. In *V. anguillarum*, RNA-α is the negative regulator of iron transporter genes, and it is preferentially expressed in the iron-rich condition [7]. These results suggest a possibility that there are other regulatory pathways involved in the regulation of *vvs*.

Involvement of NRPS is a hallmark in siderophores synthesis in bacteria (for a review, see [8]). In this study, we showed that NRPS is also involved in the siderophore production by *V. vulnificus*. Considering the fact that iron utilization is essential for survival and propagation for nearly all living organisms, especially for pathogens that survive in an iron-limiting host environment, siderophore-biosynthesis systems must be well conserved. In many bacteria, multiple genes have been identified for siderophore production. In related *Vibrio* spp., more than two genes have been identified to encode siderophores [8]. In *V. vulnificus*, null mutation in *vvsA* and *vvsB* still retained a basal level of siderophore production, suggesting that there might be other genes in addition to *vvsA* and *vvsB* for siderophore production in *V. vulnificus*. These functions need to be elucidated in future studies.

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