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비브리오 패혈증 예방을 위한 철착화제의 유용성

조선대학교대학원
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Potential Usefulness of Iron-chelating Therapy for the Prevention of *Vibrio vulnificus* Septicemia

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목 차

도목차

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한글초록

비브리오 패혈증 예방을 위한 철착화제의 유용성

박 용 진

(지도교수: 신 성 희)

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연구배경 및 목적) 패혈증 비브리오균은 그람음성 호염성 세균으로 다양한 철흡수기전들을 가지고 있음에도 불구하고 혈청 또는 조직내 철농도가 높아진 환자에게서 치명적인 패혈증을 유발하는 것으로 알려져 있다. 따라서 본 연구에서는 첫째로 실재로 비브리오 패혈증 환자에서 혈청 철농도와 철포화도가 상승되어 있는지 확인하고 둘째로 패혈증 비브리오균의 철요구량을 측정함으로써 호철성(ferrophilic) 특성이 있는지를 알아보고자 하였고 셋째로 철농도가 패혈증 비브리오균의 독력인자들의 발현에 미치는 영향을 관찰하고자 하였다. 네번째로 비브리오 패혈증의 예방을 위해 철착화제의 이용가능성을 평가하고자 하였다.

방법 및 결과) 첫째로 비브리오 패혈증 환자의 혈청에서 철농도와 트렌스페린의 농도를 직접 측정하여 정상인과 비교하였다. 혈청 철농도와 트렌스페린 철포화도는 정상인(n=45)에 비해 비브리오 패혈증 환자(n=33)에서 의미있게 높았다($p<0.05$). 혈청 트렌스페린 농도는 정상인에 비해 비브리오 패혈증 환자에서 의미있게 낮았다($p<0.05$). 둘째로 패혈증 비브리오균의 호철성(ferrophilic) 특성을 규명하였다. 패혈증 비브리오균은 증식을 시작하기 위해 트렌스페린에 결합된 철보다는 결합되지 않은 철을 더 잘 이용하였다. 또한, 황색포도알균이나 대장균에 비해 패혈증 비브리오균은 증식하기 위해 더 높은 농도의 트렌스페린과 결합하지 않은 철을 필요로 하였고, 더 적은 양의

시데로포아를 생산하였으며 트렌스페린에 결합된 철을 이용하는 능력도 낮았다. 게다가 약 1 x 10^3 cfu/ml, 즉 인체 감염시 추정되는 균수를 배지에 접종하였을 때 패혈증 비브리오균은 트렌스페린에 결합된 철을 이용하지 못하였다. 그러나 낮은 농도일지라도 트렌스페린에 결합되지 않은 철이 공급된 경우에는 트렌스페린에 결합된 철을 이용할 수 있었다. 셋째로 철농도가 패혈증 비브리오균의 독력인자들의 발현에 미치는 영향을 관찰하였다. 철은 패혈증 비브리오균으로부터 독력인자인 세포용해소/용혈소와 단백분해효소의 생산을 촉진하였다. 네번째로 비브리오 패혈증의 예방을 위해 철착화제의 이용가능성을 평가하였다. 최근에 새롭게 개발된 철착화제인 Ferriprox®에 의해서 패혈증 비브리오균의 증식이 억제되었으나 지금까지 사용되어 왔던 표준 철착화제인 Desferal®에 의해서는 패혈증 비브리오균의 증식이 오히려 촉진되었다. Desferal을 이용할 수 있는 외막단백질 즉, Desferal 수용체를 암호화하고 있는 유전자 *desA*가 사용된 모든 패혈증 비브리오 임상분리 균주(n=10)에서 발견되었고 한 균주를 제외한 나머지 환경분리 균주(9 of 10)에서도 발견되었다. 유전자 *desA*를 가지고 있는 모든 균주들은 Desferal에 의해 증식이 촉진되었고 유전자 *desA*를 가지고 있지 않은 한 균주는 Desferal에 의해 증식이 억제되었다. 유전자 *desA*의 발현은 철농도가 낮고 Desferal이 존재하는 경우에만 관찰되었고 철농도가 높은 환경이나 철농도가 낮더라도 Desferal이 존재하지 않는 경우에는 관찰되지 않았다. 유전자 *desA*를 결손시킨 돌연변이 균주의 증식은 Desferal이 존재하는 환경에서도 촉진되지 않았으며 유전자 *desA*를 회복시킨 균주의 증식은 Desferal이 존재하는 환경에서 촉진되었다. Desferal과는 대조적으로 Ferriprox는 사용된 모든 패혈증 비브리오 균주들의 증식을 억제할 수 있었다.

결론 및 제언) 패혈증 비브리오균은 다른 세균들에 비해 증식하기 위해 높은 농도의 트렌스페린 결합되지 않은 철을 필요로 할 뿐만아니라 트렌스페린에 결합된 철보다 트렌스페린에 결합되지 않은 철을 선호하는 호철성 세

균이기 때문에 혈청 또는 조직내 철농도가 상승된 환자에게서 치명적인 패혈증을 유발하는 것으로 생각된다. 또한 높아진 철농도는 패혈증 비브리오균의 독력인자들의 발현을 촉진함으로써 패혈증의 경과를 악화시킬 수 있는 것으로 판단된다. 이러한 사실에 근거하여 패혈증 비브리오균이 이용하기 쉬운 가용철농도를 낮춤으로써 균증식을 억제할 수 있는 Ferriprox를 이용한 철착화요법은 비브리오 패혈증 예방을 위한 유용한 방법이 될 수 있을 것으로 판단된다. 또한 Desferal이 존재하는 환경에서만 발현되는 Desferal 수용체가 임상 분리균주들 뿐만 아니라 환경 분리균주에서도 존재하고 있다. 따라서 철착화제인 Desferal을 사용하여 철과부하를 개선하는 치료방법은 오히려 치명적인 비브리오 패혈증을 초래할 수 있는 위험요인이 될 수 있다.

핵심어: 패혈증 비브리오균, 호철성 세균, 철, 철착화치료법, Desferal (Deferoxamine), Ferriprox (Deferiprone)

1. Introduction

Vibrio vulnificus is a gram-negative halophilic and alkalophilic bacterium that causes fatal septicemia, especially in patients with underlying diseases such as liver cirrhosis and alcoholic hepatitis as the result of ingestion of raw seafood contaminated by the bacterium or of wound infections by the bacterium. *V. vulnificus* septicemia (VVS) rapidly progresses with a fulminant course and eventually shows a mortality rate exceeding 50% despite aggressive therapy [Park *et al*., 1991;Chuang *et al*., 1992].

Several potential virulence factors have been associated with VVS, including polysaccharide capsule, lipopolysaccharide, metalloprotease (VvpE), hemolysin or cytolysin (VvhA), RtxA toxin, phospholipase, motility, and iron-assimilation systems [Linkous & Oliver, 1999; Strome & paranjpye, 2000; Gulig *et al*., 2005]. Among these factors, VvhA and VvpE have been the most extensively studied. VvhA has proved to be the most potent exotoxin produced by *V. vulnificus*. VvhA kills mice, exhibits hemolytic or cytolytic activity [Gray & Kreger, 1985; Rhee *et al*., 1994; Lee *et al*., 2004]. In animal models, locally and systemically administered VvhA reproduces the same clinical and pathological manifestations of septicemia as caused by the administration of live bacteria [Gray & Kreger, 1987; Rhee *et al*., 1994; Park *et al*., 1996]. Moreover, VvhA induces vasodilation and hemolysis or cytolysis at a very low level [Kook *et al*., 1996 & 1999], stimulates the production of inflammatory cytokines including TNF-α [Park *et al*., 1996 & 1998], and induces superoxide anion-mediated apoptosis in human vascular endothelial cells [Kwon *et al*., 2001]. Purified

VvpE has been shown to induce hemorrhagic damage and dermonecrosis, enhance vascular permeability and edema, and has also proven lethal to mice [Kothary & Kreger, 1987; Molla *et al*., 1989; Miyoshi & Shinoda, 1988 & 1992; Maruo *et al*., 1998; Shao & Hor, 2001; Hülsmann *et al*., 2003; Jeong *et al*., 2003; Jeong *et al*., 2003; Kim et al., 2003; Kim *et al*., 2005]. Production of VvhA and VvpE is known to be regulated by several environmental factors [Paranjpye *et al*., 1998; Bang *et al*., 1999; Lee *et al*., 2000; Choi *et al*., 2002; Kim *et al*., 2003]. Nevertheless, only a few reports are available on the effect of iron on the expression of *V. vulnificus* virulence factors [Kawase *et al*., 2004; Shin *et al*., 2005].

Iron availability within the human body is very low despite large amounts of iron being present. Over 99% of iron is sequestered within cells as hemoglobin, myoglobin or ferritin-hemosiderin. Moreover, most extracellular iron is tightly bound to high-affinity iron-binding glycoproteins, such as transferrin and lactoferrin. Accordingly, the level of freely available iron within the human body is too low to sustain growth [Neilands, 1991; Weinberg & Weinberg, 1995] although iron is essentially required for the survival and proliferation of most bacteria. Under some pathologic conditions, iron-availability increases as intracellular iron is released due to cell destruction or the elevation of transferrin iron saturation levels. This increased iron availability increases host susceptibility to a variety of opportunistic pathogens including *V. vulnificus* [Weinberg & Weinberg, 1995; Bullen *et al*., 2005].

V. vulnificus can utilize both transferrin-bound-iron (TBI) and non-transferrin-bound-iron (NTBI) including hemoglobin and ferritin via the siderophore-mediated and heme receptor-mediated iron-uptake systems

[Simpson & Oliver, 1987; Litwin & Byrne, 1998]. *V. vulnificus* is known to produce catechol (phenolate)- and hydroxamate-siderophores for iron-acquisition under iron-limited conditions [Simpson & Oliver, 1983]. Catechol-siderophore (called vulnibactin) is known to play a more important role in iron-acquisition from TBI or NTBI than hydroxamate-siderophore [Litwin *et al*., 1996; Webster & Litwin, 2000]. *V. vulnificus* is also known to express a heme-specific receptor for direct iron-acquisition from heme proteins [Litwin & Byrne, 1998]. In addition, it was recently found that the DesA- and IutA-mediated iron-uptake systems are capable of utilizing heterologous siderophores, *Streptomyces pilosus* deferoxamine and *Escherichia coli* aerobactin, respectively [Takata *et al*., 2005; Tanabe *et al*., 2005].

Elevation of serum iron levels is the best-known predisposing host factor of VVS [Wright *et al*., 1981; Brennt *et al*., 1991; Bullen *et al*., 1991; Hor *et al*., 1998; Starks *et al*., 2000]. Nevertheless, to date, there is no report that directly measures serum iron levels in VVS patients. More controversially, *V. vulnificus* preferentially causes fatal septicemia in patients with elevated serum iron levels although it possesses several efficient iron-uptake systems, as described above. This controversy suggests that *V. vulnificus* may be a ‘ferrophilic’ bacterium that requires higher levels of available iron than other pathogens for growth initiation. However, to date, there is no report that measures the iron-requirement of *V. vulnificus.*

Bacterial iron-uptake systems have been attractive targets for the development of preventive or therapeutic vaccines. In the similar regard, iron-chelation therapy capable of limiting bacterial iron-uptake has also re-

ceived some attention as attractive novel preventive or therapeutic means [Kontoghiorghes & Weinberg, 1995; Brown *et al*., 2001; von Bonsdorff *et al*., 2001; Etz *et al*., 2002; Marx, 2002].

Deferoxamine (Desferal®; $C_{25}H_{48}N_6O_8 \bullet CH_4O_3S$; called desferrioxamine) is the best known hydroxamate siderophore derived from *Streptomyces pilosus* and has been used for long time as the standard parenteral iron-chelator for the treatment of iron-overload [Olivieri, 1997]. Desferal has been known to exhibit antimicrobial potential, mainly because it can compete with bacteria for available iron [van Asbeck *et al*., 1983]. *In vitro* studies have demonstrated that Desferal has bacteriostatic activity against some pathogenic bacteria especially in the presence of ascorbic acid [van Asbeck *et al*., 1983; Hartzen, *et al*., 1989, 1991 and 1994]. However, one of the drawbacks of Desferal is that some pathogenic bacteria including *Yersinia enterocolitica* and *Staphylococcus aureus* are capable of utilizing the drug for iron-uptake just like their specific siderophores via specific receptors [Baumler & Hanke, 1992; Sebulsky *et al*., 2000; Lesic *et al*., 2002]. More recently, a putative Desferal-receptor DesA was found in a *V. vulnificus* strain [Aso *et al*., 2002; Tanabe *et al*., 2005]. However, the function of DesA as the Desferal-receptor has not been confirmed yet in accordance with the molecular version of Koch's postulates [Falkow, 2004], and the ubiquitousness of DesA in *V. vulnificus* clinical and environmental isolates has not been verified.

Recently, a new synthetic oral iron-chelator, deferiprone (Ferriprox®; 1,2-dimethyl-3-hydroxypyrid-4-one; called L1), was clinically available [Hoffbrand *et al*., 1998; Barman Balfour & Foster, 1999; Kontoghiorghes, 2006]. Ferriprox forms strong complexes with iron in the

ratio of three to one at physiological pH, and can capture iron from transferrin, lactoferrin, ferritin and hemosiderin, *in vivo* as well as *in vitro*, the effect being greater than that of parenteral Desferal. Moreover, Ferriprox has been known to inhibit even the growth of bacteria capable of utilizing Desferal because its structure is completely different from that of Desferal.

In this study, first, we directly measured serum iron levels in *V. vulnificus* septicemia patients in order to confirm whether or not serum iron levels are elevated in *V. vulnificus* septicemia patients, secondly measured the iron-requirement of *V. vulnificus* in order to determine the ferrophilic characteristic of *V. vulnificus,* thirdly, determined the effect of elevated iron on the production of hemolysin/cytolysin (VvhA) and metalloprotease (VvpE), evaluated the potential usefulness of the two clinically-available iron-chelators, Desferal and Ferriprox, for the prevention and treatment of VVS, and finally examined the ubiquitousness of DesA in *V. vulnificus* clinical and environmental isolates.

2. Materials and Methods

2.1. Patients' sera

Septicemic serum samples were taken from patients suspected of having VVS following the informed consent in the emergency room of Chonnam National University Hospital from 1980, and were stored at -70^{o}C. Of these septicemic sera, thirty-three serum samples obtained from VVS patients, who showed typical clinical manifestations and culture positivity, were used in the study. All VVS patients were ≥ 50-year-old males with underlying liver cirrhosis or a heavy alcohol drinking habit, as previously reported [Shin *et al*., 2002]. Normal control (NC) serum samples (n=45) were obtained from normal healthy ≥ 50-year-old males who had received regular medical examinations following the informed consent at Chosun University Hospital in 2001, and were stored at -70^{o}C.

2.2. Measurement of serum iron indices and transferrin concentrations

All stored VVS and NC sera were thawed, and serum iron indices were measured. Four serum iron indices, i.e., total iron concentration (TIC), unbound iron-binding capacity (UIBC), total iron-binding capacity (TIBC), and transferrin iron-saturation (IS), were measured using the Iron and Total Iron-Binding Capacity Kit (Sigma, MO, USA). Serum transferrin levels were measured using the competitive ELISA method. Briefly, ELISA plate wells were coated with 100 μl of coating buffer (0.05 M carbonate buffer, pH 9.6) containing goat-anti-human transferrin (1:500; Jackson ImmunoResearch Laboratories Inc., PA, USA) by incubating the plates at 37^{o}C for 1 h. Plates were then washed three times with wash-

ing buffer [PBS (pH 7.2) containing 0.05% Tween-20]. Human transferrin conjugated with horseradish peroxidase (1:500 in PBS containing 1% bovine serum albumin; Jackson ImmunoResearch Laboratories Inc.) was mixed with sera (1:50 in PBS containing 1% bovine serum albumin), and a 100 μl aliquot of this mixture was added to the pre-coated wells. The plates were then incubated at 37°C for 1 h, and then washed. O-phenylenediamine (Sigma) was used as the substrate of peroxidase, and absorbance was measured at 450 nm. To obtain a reference curve, a two-fold diluted apotransferrin solutions (Sigma) were used. The serum iron indices and transferrin concentrations of each VVS patient and NC person were expressed as the averages of duplicate determinations. Statistical significances were determined using the Mann-Whitney Rank Sum test and the Pearson Correlation test.

2.3. Preparation of cirrhotic ascites (CA)

Five CA samples were obtained from five patients in keeping with the therapeutic purpose following the informed consent at the Chosun University Hospital as described in our previous study [Choi *et al*., 2006]. All five patients had been diagnosed with liver cirrhosis by biopsy. The CA samples were immediately stored at -25°C. Immediately prior to use, the samples were thawed and equal volumes of the five CA samples were mixed to abolish individual difference of CA constituents, and the pooled CA was filtered with 0.45 μm pore-sized disposable filters for sterilization and cell removal. The pooled CA was heat-inactivated at 65°C for 30 min in order to inactivate possible bactericidal components, including complement.

2.4. Bacterial strains, media and reagents

Bacterial strains, plasmids and primers used in this study were listed in Table 1, 2 and 3. Heart Infusion (HI; BD, MD, USA) broth with additional 2% NaCl was used to cultivate *V. vulnificus,* and HI broth alone to cultivate *S. aureus* and *E. coli.* HI broth was deferrated using 8-hydroxyquinoline (Sigma), as described by Leong and Neilands [Leong & Neilands, 1982]. The residual iron concentration of deferrated HI (DF-HI) broth was approximately 1 μM. When necessary, human holotransferrin (HT; 1,200~1,600 μg of iron per 1 g of protein; Sigma), partially iron-saturated transferrin (PT; 300~600 μg of iron per 1 g of protein; Sigma) or ferric chloride (FC; Sigma) were used as iron sources. Human apotransferrin (AT; less than 30 μg of iron per 1 g of protein; Sigma), α,α'-dipyridyl (DP; Sigma), Desferal® (CIBA-GEIGY, Switzerland) and Ferriprox® (Apotex Inc., Canada) were used as iron-chelators.

2.5. Construction of a *desA*-deletion mutant

An in frame-deletion mutant of the *V. vulnificus desA* gene was constructed by crossover PCR using the suicide vector pDM4 with R6K origin [McGee *et al*., 1996]. Two pairs of PCR primer set (DesA-up-1/DesA-up-2 and DesA-down-1/DesA-down-2) were used for the PCR amplification of the 5' and 3' fragments of the *V. vulnificus desA* gene. The two PCR products, 788 bp and 1,047 bp were used as a template for a second PCR amplification using the PCR primer set, DesA-up-1 with *Bam*HI overhang and DesA-down-2 with *Spe*I overhang.

The 1,835 bp *Bam*HI-*Spe*I fragment with the deletion of the *desA* internal sequences was cloned into the *Bgl*II-*Spe*I site of pDM4. The resulting plasmid pRC102 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [Miller & Mekalanos, 1988], and subsequently transferred to *V. vulnificus* MO6-24/O strain by conjugation. Transconjugants were selected on TCBS agar containing chloramphenicol, and then stable transconjugants were spread onto HI agar containing 10% sucrose to allow the second homologous recombination to occur. The resulting deletional mutation in the *V. vulnificus desA* gene (RC102) was confirmed by PCR (data not shown).

2.6. *In trans* complementation of the *desA* gene

To restore the wild type *desA* gene in the *desA*-deletion mutant RC102 strain, a 3.99 kb *Bam*HI-*Hin*dIII fragment containing the 2,181 bp *desA* gene was amplified using the PCR primer set, DesA-up-1 with *Bam*HI overhang and DesA-down-3 with *Hin*dIII overhang, and subsequently subcloned into the broad host range vector pLAFR3II (named pRC106), which had been prepared by subcloning a 1,453 bp *Bam*HI-*Bgl*II fragment containing the *bla* gene of pUTKm1 into the *Bgl*II site of pLAFR3 [Herreo *et al*., 1990; Staskawicz *et al*., 1987]. The pRC106 was transferred into the *desA*-deletion mutant RC102 strain via triparental mating using a conjugative helper plasmid pRK2013 [Ditta *et al*., 1980]. The transconjugants were screened on TCBS agar plates containing ampicillin and tetracycline. The presence of the wild type allele in the *V. vulnificus desA*-deletion mutant strain was confirmed by PCR using the primer set, DesA-up-1 and DesA-down-3 (named RC106).

2.7. Growth conditions and measurements

V. vulnificus strains were cultured overnight in HI broth containing 100 μM dipyridyl at 37°C in order to adapt them to iron-limited conditions and to reduce intracellular iron storages. These preconditioned strains in the late exponential growth phase were then inoculated into test media. The initial bacterial inoculum was adjusted to concentrations of about 1 x 10^6 or 1 x 10^3 colony-forming units (cfu)/ml according to the experiment, and cultured with vigorous shaking (200 rpm) for 12 or 24 h at 37°C. During culture, culture aliquots were withdrawn at the indicated times. Bacterial growth was monitored by measuring the OD_{600} values of the culture aliquots. Bacterial growths are expressed as the means and standard errors of the OD_{600} values measured in triplicate. Culture supernatants were also obtained by the centrifuge of culture aliquots for 5 min at 10,000 rpm.

2.8. Measurement of hemolysin/cytolysin and metalloprotease

The hemolytic activity in culture supernatants was measured in triplicate by the conventional tube hemolysis assay using 1% human RBC suspension as described by Fan *et al*. [2001]. In brief, 100 μl of culture supernatants were mixed with 400 μl of 1% RBC suspension, and the mixtures were then incubated at 37°C. After incubation for 1 h, unlyzed RBC and cell debris were removed by centrifugation, and then the OD_{540} values of the resulting supernatants was measured. Hemolytic activity (%) was expressed as the OD_{540} value of specimen/ the OD_{540} value of complete hemolysis by Triton X-100 x 100. In order to measure extracellular

VvpE production, we measured caseinolytic activity in culture supernatants. The caseinolytic activity was measured as described previously [Jeong *et al*., 2000 & 2001] without modification.

2.9. Siderophore assays and 6 M urea-gel electrophoresis

Chrome azurol S (CAS) agar diffusion assay was used to determine total siderophore production in culture supernatants [Shin *et al*., 2001]. In brief, total 90 μl of culture supernatants were loaded into the wells formed in CAS agar and plates were incubated overnight at 37^{o}C. The formation of orange haloes around wells indicated total siderophore activity. Transferrin molecules are separated into four forms on 6 M urea-gel, i.e., apo- (AP), C-terminal monoferric (MC), N-terminal monoferric (MN), and diferric (DF) forms, according to their iron-saturated levels. Accordingly, 6 M urea-gel electrophoresis was performed to monitor iron-removal from transferrin during culture, as described by Makey and Seal [Makey & Seal, 1976]. In brief, 20 μl aliquots of culture supernatants were mixed with urea-gel sample buffer containing 8 M urea, but not SDS or mercaptoethanol, and allowed to react at 37^{o}C for 30 min without heating. Mixtures were then electrophoresed on a 5% stacking and 6% running urea-gel. Proteins were visualized by Coomassie blue staining.

2.10. PCR and reverse transcriptase (RT)-PCR

For PCR, *V. vulnificus* strains were inoculated into HI broths at 1 x 10^{6} cfu/ml, and cultured with vigorous shaking for 6 h at 37^{o}C. Culture aliquots were mixed well with lysis buffer (1 mM EDTA and 0.5% Triton), boiled for 10 min, and centrifuged at 13,000 rpm for 5 min to remove cell

debris and obtain supernatants. The supernatants containing template genomic DNA were mixed with PCR premix (iNtRon Biotechnology, Korea) containing the DesA-1 and 2 primer set. Total 30 cycles of PCR were performed under the following conditions: 94^{o}C for 2 min for pre-denaturation, 94^{o}C for 20 sec for denaturation, 48^{o}C for 10 sec for annealing, 72^{o}C for 40 sec for extension, and 72^{o}C for 5 min for post-extension (Mastercycler Gradient, Eppendorf, Germany). Amplified products were electrophoresed on agarose gels and stained with ethidium bromide.

For RT-PCR, *V. vulnificus* strains, grown in HI broths containing 100 μM dipyridyl, were inoculated into test broths at 1 x 10^{6} cfu/ml, and cultured with vigorous shaking at 37^{o}C for 6 h. Bacterial pellets were obtained by centrifuging culture aliquots at 10,000 rpm for 5 min. Total RNA was isolated from the bacterial pellets using Trizol® reagent (Molecular Research Center, USA). After treatment with DNase (Qiagen), 200 ng of total RNA was reverse-transcribed to make cDNA using a random primer (Promega) and then amplified using PCR Premix (iNtRon Biotechnology) and the *desA*- and *vuuA*-specific primer sets (DesA-3 and 4; VuuA-1 and 2) or the 16s rRNA-specific primer set (16s rRNA-1 and 2) as the control. Total 30 cycles of PCR were performed under the following conditions: 94^{o}C for 5 min for pre-denaturation, (94^{o}C for 30 sec for denaturation, 60^{o}C for 30 sec for annealing, 72^{o}C for 30 sec for extension), and 72^{o}C for 5 min for post-extension. Amplified products were electrophoresed on agarose gels and stained with ethidium bromide.

Table 1. Bacterial strains used in this study.

Strains	Characteristics	Sources or references
V. vulnificus		
MO6-24/O	Highly virulent clinical isolate	Our lab. stock
RC102	MO6-24/O with *desA*-deletional mutation	This study
RC106	RC102 with *desA*-complementation	This study
E1~E10	Environmental isolates (n=10) showing opaque colony on HI agar	Our lab. stock
C1~C10	Clinical isolates (n=10) showing opaque colony on HI agar	Our lab. stock
S. aureus		
*ATCC6539	Clinical isolate	-
Others	Clinical isolates (n=10)	Our lab. stock
E. coli		
ATCC25922	Clinical isolate	-
Others	Clinical isolates (n=10)	Our lab. stock
SY327 λ*pir*	Δ(*lac pro) argE*(Am) *rif nalA recA56* λ*pir* lysogen; Host for suicide vector *thi thr leu tonA lacY supE*	Miller & Mekalanos, 1998
SM10 λ*pir*	*recA*::RP4-2-Tc^r::Mu Km^r λ*pir* lysogen: Conjugation donor	Miller & Mekalanos, 1998

*ATCC: American Type Culture Collection

Table 2. Plasmids used in this study.

Plasmids	Characteristics	Sources or references
pDM4	Suicide vector with R6K origin; Cm^r	McGee *et al*., 1996
pCMM230	PCR-amplified truncated *vis* gene (0.67-kb) cloned into pCR®2.1-TOPO®	This study
pCMM232	*Xba*I-*Hin*dIII fragment of pCMM230 cloned into pNQ705	This study
pRC102	1.835-kb *Bam*HI-*Spe*I fragment containing an in-frame deletion of *V. vulnificus desA* gene cloned into pDM4	This study
pRC106	3.99-kb *Bam*HI-*Hin*dIII fragment containing *desA* gene cloned into pLAFR3II	This study
pLAFR3II	pLAFR3 with *bla* inserted at the cos site; Ap^r Tc^r	This study
pLAFR3	IncP cosmid vector; Tc^r	Herreo *et al*., 1990
pUTKm1	Tn5-based insertion delivery plasmid, Ap^r	Staskawicz *et al*., 1987
pRK2013	IncP, Km^r, Tra $Rk2^+$ *repRK2 repE1*	Ditta *et al*., 1980

Table 3. Primers used in this study.

Primers	Sequences	Sources or references
DesA-1	5’-cccaacttgaaaccattacg-3’	This study
DesA-2	5’-ggtgataagtgttctcttgg-3’	This study
DesA-3	5’-atcccatgataagacaagtacagcg-3’	This study
DesA-4	5’-accttgagacgttagttcgcccaattgg-3’	This study
DesA-up-1	5’-cgggatccgcctttagccgcgcattcaagcag-3’	This study
DesA-up-2	5’-tggttagaagcccatagtgtccctaaataccaatg-3’	This study
DesA-down-1	5’-agggacactatgggcttctaaccaaataaaatcgac-3’	This study
DesA-down-2	5’-gactagtgcgcgcgaaaattcttgagctcg-3’	This study
DesA-down-3	5’-cccaagcttgcgcgcgaaaattcttgagctcg-3’	This study
Vis-1	5’-gccaatcaggaactctcg-3’	This study
Vis-2	5’-ctccagcaaacaccttcatc-3’	This study
VuuA-1	5’-gcctatgctcaaaccgagag-3’	This study
VuuA-2	5’-accaacacctgcttttaccg-3’	This study
16s rRNA-F	5’-aacgagcgcaacccttatcc-3’	This study
16s rRNA-R	5’-actccaatggactacgacgcac-3’	This study

3. Results

3.1. Serum total iron concentrations and transferrin iron-saturations in VVS patients

When correlations between the four serum iron indices were analyzed for all sera (n=78) sampled in this study. TIC was found to be positively correlated with IS ($R=0.52$, $p<0.05$), and IS to be negatively correlated with UIBC ($R=-0.83$, $p<0.05$). The means and standard deviations of TIC, UIBC, TIBC and IS in VVS sera (n=33) were 103.2 ± 123.4 μg/dl, 109.1 ± 68.4 μg/dl, 212.4 ± 121.5 μg/dl and 47.9 ± 25.2%, respectively. Of these four indices, only TIC and IS were 2.1- and 1.7-fold increased in the VVS sera, respectively, and these increases were significant versus NC sera ($p<0.05$). The distributions of TIC and IS in VVS sera and NC sera are shown in Figure 1AB. TIC and IS values in the VVS sera were ranged from 4.7 to 747.7 μg/dl and from 1.7 to 100%, respectively. These values were higher than the mean values of NC sera in 76% and 79% of VVS sera, respectively.

3.2. Serum transferrin concentrations in VVS patients

Transferrin concentrations in NC sera were well correlated with TIBC ($R=0.63$, $p<0.05$), but no correlation was found in VVS sera ($R=0.05$, $p>0.05$). The means and standard deviations of transferrin concentrations in the NC and VVS patient sera were 1287.7 ± 662.8 μg/ml and 452.8 ± 367.9 μg/ml, respectively, and this decrease was significant ($p<0.05$). The distributions of transferrin concentrations in the VVS and

NC sera are shown in Figure 2. Transferrin concentrations in the VVS sera ranged from 71.4 to 1,381.2 μg/ml. Transferrin concentrations were lower than the mean values of the NC sera in 94% of the VVS sera.

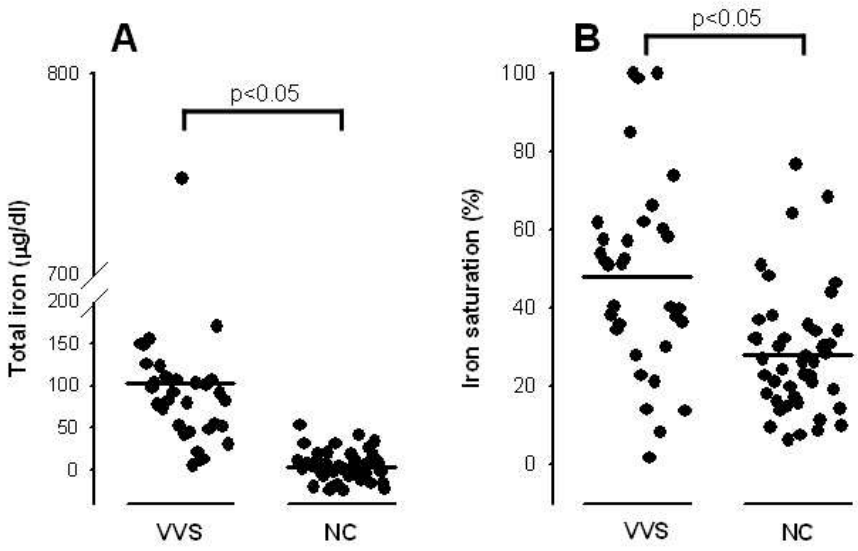


Figure 1. Elevated serum total iron (A) and transferrin iron-saturation (B) levels in *Vibrio vulnificus* septicemia (VVS) patients (n=33) versus normal controls (NC; n=45). Serum total iron and transferrin iron-saturation levels were determined using the Iron and Total Iron-Binding Capacity Kit. The levels in each VVS patient or NC person are expressed as the averages of duplicate determinations. The solid bars indicate the mean values for all VVS patients and NC persons. The statistical significance of data was analyzed using the Mann-Whitney Rank Sum test.

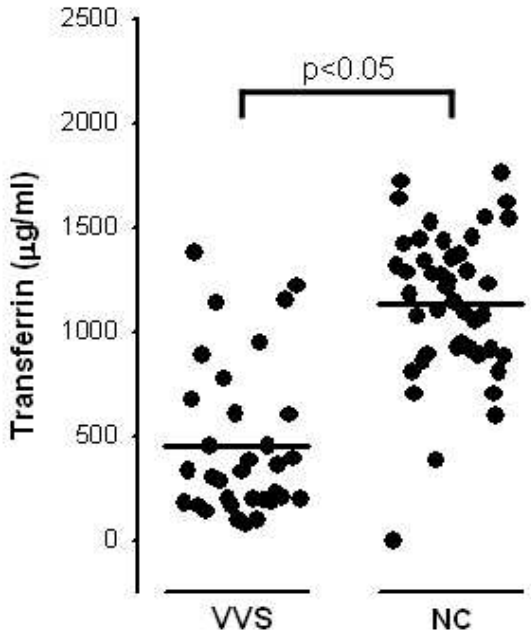


Figure 2. Decreased serum transferrin concentrations in *Vibrio vulnificus* septicemia (VVS) patients (n=33) versus normal controls (NC; n=45). Serum transferrin levels were determined by competitive ELISA. Two-fold diluted apotransferrin solutions were used to obtain a reference curve. The transferrin concentration of each VVS patient or NC person is expressed as the averages of duplicate determinations. The solid bars indicate the mean values for all VVS patients and NC persons. The statistical significance of data was analyzed using the Mann-Whitney Rank Sum test.

3.3. The relative iron requirement of *V. vulnificus*

Bacterial iron requirements for growth initiation may vary among species or strains and be dependent on culture conditions. Accordingly, we compared the relative iron requirement of *V. vulnificus* for growth initiation with those of *E. coli* and *S. aureus* in HI broth which is one of the most appropriate media for *V. vulnificus* cultivation [Kim *et al*., 2003]. Initial bacterial densities were all approximately 1 x 10^6 cfu/ml. The growths of *V. vulnificus, E. coli* and *S. aureus* were inhibited dose-dependently in HI broth containing various concentrations of DP (~0.5 mM), but *V. vulnificus* grew in HI broth containing lower concentrations of DP than *E. coli* and *S. aureus* (Figure 3A). Similar results were observed in HI broth containing various concentrations of AT (~2.0 mg/ml) (Figure 3B). We also observed a similar trend when the initial bacterial densities of the three bacteria were reduced to approximately 1 x 10^3 cfu/ml (data not shown). These results indicate that *V. vulnificus* does not utilize DP-bound iron or AT-bound iron as effectively as *E. coli* or *S. aureus*; that is to say, *V. vulnificus* requires a higher level of freely-available iron for growth initiation than *E. coli* or *S. aureus.*

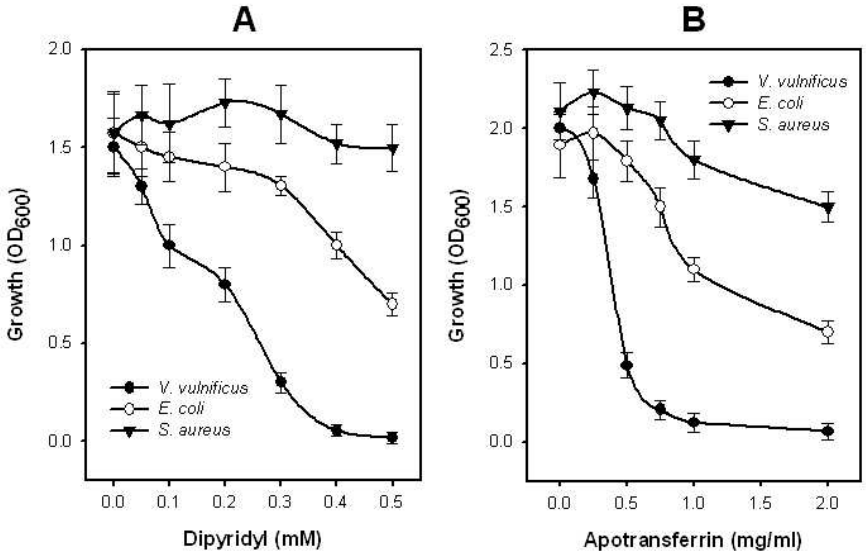


Figure 3. Relative iron-requirements of *Vibrio vulnificus*. *V. vulnificus* MO6-24/O strain, *E. coli* ATCC25922 strain and *S. aureus* ATCC6538 strain grown in HI broth containing 100 μM dipyridyl were inoculated into HI broths containing various concentrations of dipyridyl (A) and apotransferrin (B) at a concentration of 1 x 10^6 cfu/ml and cultured with vigorous shaking at 37°C for 12 h. Bacterial growths were monitored by measuring the OD_{600} values of culture aliquots at 12 h, and are expressed as the means and standard errors of the OD_{600} values measured in triplicate.

3.4. The relative ability of *V. vulnificus* to produce siderophores and utilize TBI

In order to compare the relative abilities of the three strains to produce siderophores and utilize TBI, we cultured *V. vulnificus* MO6-24/O strain, *E. coli* ATCC25922 strain and *S. aureus* ATCC6538 strain in DF-HI broth and in DF-HI broth containing 0.5 mg/ml HT at an initial bacterial density of 1 x 10^6 cfu/ml. The growths of the three bacteria were stimulated by adding HT (Figure 4A). On comparing the abilities to produce siderophores in DF-HI broth using CAS agar diffusion assays, *V. vulnificus* was found to produce smaller amounts of siderophores than *E. coli* or *S. aureus* (Figure 4B). On comparing the abilities to utilize TBI in DF-HI containing HT by 6M urea-gel electrophoresis, *V. vulnificus* was found to utilize iron from HT less efficiently than *E. coli* or *S. aureus* (Figure 4C). A large portion of transferrin bands was clearly shifted up from DF-and MN-forms to MC- or AP-form in *E. coli* and *S. aureus,* but only a small portion of transferrin bands was shifted in *V. vulnificus*. Similar results were observed in all clinical isolates (n=10) of *V. vulnificus*, *E. coli* and *S. aureus* (Figure 5AB). In addition, *E. coli* and *S. aureus*, but not *V. vulnificus*, grew actively when PT was the sole iron source (data not shown). Overall, these results indicate that *V. vulnificus* cannot assimilate iron from transferrins as effectively as *E. coli* or *S. aureus* because of its relative inability to produce siderophores.

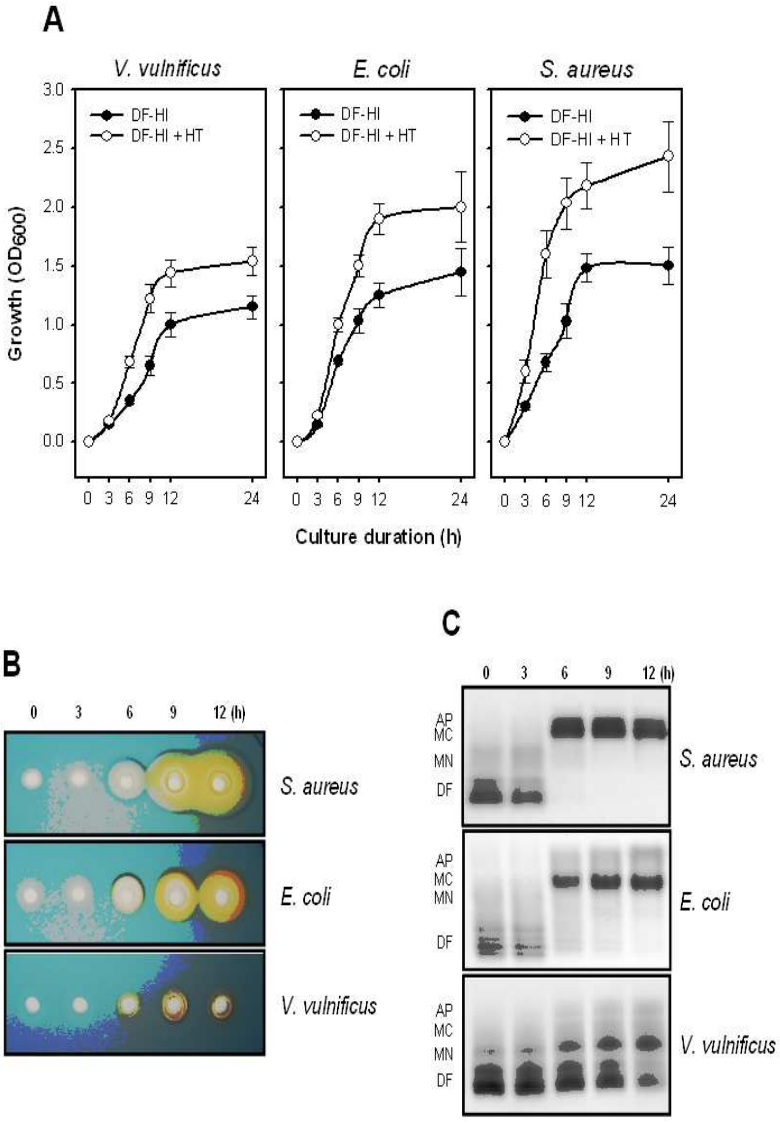

A
V. vulnificus
E. coli
S. aureus
DF-HI
DF-HI + HT
Growth (OD600)
Culture duration (h)
B
0 3 6 9 12 (h)
S. aureus
E. coli
V. vulnificus
C
0 3 6 9 12 (h)
AP
MC
MN
DF
S. aureus
E. coli
V. vulnificus

Figure 4. The ability of *Vibrio vulnificus* to produce siderophores and to utilize transferrin-bound-iron. *V. vulnificus* MO6-24/O, *S. aureus* ATCC6538 and *E. coli* ATCC25922 strains, grown in HI broth containing 100 μM dipyridyl, were inoculated into deferrated (DF) HI broth or DF-HI broth containing 0.5 mg/ml holotransferrin (HT) at 1 x 10^6 cfu/ml and cultured with vigorous shaking at 37°C for 24 h. (A) Bacterial growths were monitored by measuring the OD_{600} values of culture aliquots at the indicated times, and are expressed as means and standard errors of the OD_{600} values measured in triplicate. Culture supernatants were obtained to measure total siderophore activity (B) and iron removal from transferrin (C) at the indicated times by centrifuging culture aliquots. Siderophore activity was determined by CAS agar diffusion assay using 90 μl of culture supernatants. Iron removal from HT was measured by 6 M urea-gel electrophoresis using 20 μl of culture supernatants. The results shown in B and C were representative of experiments performed in triplicate.

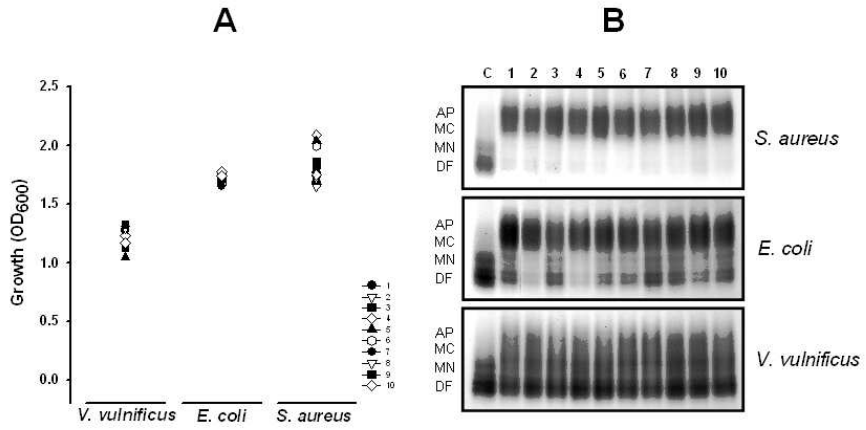


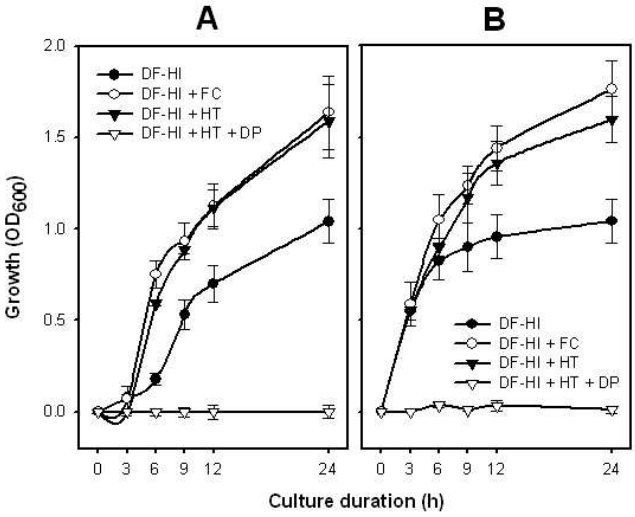
Figure 5. The ability of *Vibrio vulnificus* clinical strains to utilize transferrin-bound-iron. *V. vulnificus, S. aureus* and *E. coli* clinical strains (n=10), grown in HI broth containing 100 μM dipyridyl, were inoculated into deferrated HI broth containing 0.5 mg/ml holotransferrin at 1 x 10^6 cfu/ml and cultured with vigorous shaking at 37°C for 12 h. (A) Bacterial growths were monitored by measuring the OD_{600} values of culture aliquots at 12 h. (B) Iron removal from HT was measured by 6 M urea-gel electrophoresis using 20 μl culture supernatant aliquots.

3.5. *V. vulnificus* prefers NTBI to TBI for growth initiation

The preference of bacteria for NTBI is known to be dependent on initial bacterial densities [Matinaho *et al*., 2001]. Accordingly, in order to determine which iron source, i.e., NTBI or TBI, *V. vulnificus* prefers for growth initiation, we inoculated *V. vulnificus* MO6-24/O strain into in DF-HI broth containing 10 μM FC or 0.5 mg/ml of HT at *ca*. 1 x 10^3 or 1 x 10^6 cfu/ml, and cultured with vigorous shaking (220 rpm) at 37^{o}C for 24 h. In all broths, the growth of MO6-24/O strain was stimulated by increasing the initial bacterial density from 1 x 10^3 to 1 x 10^6 cfu/ml (Figure 6AB). Moreover, the growth of MO6-24/O strain was stimulated by adding FC regardless of initial bacterial density. Similar results were observed when the FC concentration was reduced to 5.0 μM (data not shown). However, at the initial bacterial density of 1 x 10^3 cfu/ml, MO6-24/O strain hardly removed iron from HT so that could not shift up transferrin bands, especially in the early growth phase (Figure 6C), although its growth appeared to be substantially stimulated by HT from the early growth phase (Figure 6A). This discrepancy is believed to be due to residual free iron contained during the manufacture of HT [Choi *et al*., 2006]. In contrast, at an initial bacterial density of 1 x 10^6 cfu/ml, MO6-24/O strain removed iron from HT efficiently so than could shift transferrin bands up (Figure 6C), and this began slightly later than its growth (Figure 6B). These results indicate that *V. vulnificus* prefers NTBI to TBI for growth initiation, especially at low initial bacterial densities.

DF-HI broth containing HT contains free iron in HT and a residual free iron level of *ca.* 1 μM after deferration. Accordingly, in order to fur-

ther limit free iron without changing TBI levels, we added 200 μM DP to DF-HI broth containing HT. TBI levels remained unchanged despite the addition of DP (Figure 6C), but after this addition MO6-24/O strain was unable to utilize iron from TBI or grow on TBI regardless of initial bacterial densities (Figure 6AB). These results indicate that *V. vulnificus* cannot utilize TBI as an iron source for growth initiation regardless of initial bacterial densities under severely NTBI-limited conditions. That is to say, NTBI is essentially required for efficient TBI utilization by and the growth initiation of *V. vulnificus*.


A
B
DF-HI
DF-HI + FC
DF-HI + HT
DF-HI + HT + DP
Growth (OD600)
Culture duration (h)
0 3 6 9 12 24
0.0 0.5 1.0 1.5 2.0

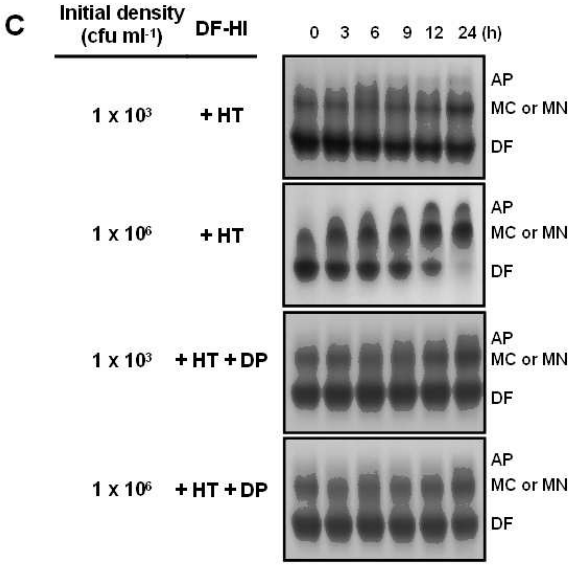

C
Initial density (cfu ml-1)
DF-HI
0 3 6 9 12 24 (h)
1 x 10³ + HT
1 x 10⁶ + HT
1 x 10³ + HT + DP
1 x 10⁶ + HT + DP
AP
MC or MN
DF

Figure 6. Non-transferrin-bound-iron requirement of *Vibrio vulnificus* for growth initiation and efficient transferrin-bound-iron utilization. *V. vulnificus* MO6-24/O strain, grown in HI broth containing 100 μM dipyridyl, was inoculated into deferrated (DF) HI broth or DF-HI broth containing 0.5 mg/ml holotransferrin (HT) or 10 μM ferric chloride (FC) or HT plus 200 μM dipyridyl (DP) at 1 x 10^3 (A) or 1 x 10^6 (B) cfu/ml, respectively, and cultured with vigorous shaking at 37°C for 24 h. Bacterial growths were monitored by measuring the OD_{600} values of culture aliquots, which were withdrawn at the indicated times. Results are expressed as the means and standard errors of OD_{600} values measured in triplicate. (C) Culture supernatants were obtained to measure iron removal from transferrin at the indicated times by centrifuging culture aliquots. Iron removal from HT was measured by 6 M urea-gel electrophoresis using 20 μl aliquots of culture supernatants. Results are representative of experiments performed in triplicate.

3.6. Iron stimulates the production of hemolysin/cytolysin and metalloprotease

In order to observe the effect of iron on the production of VvhA and VvpE, we inoculated *V. vulnificus* MO6-24/O strain in DF-HI or in DF-HI containing 10 μM FC at about 1 x 10^6 cfu/ml and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. During culture, culture aliquots were obtained at appropriate times to measure bacterial growth, and culture supernatants to observe the productions of VvhA and VvpE. The hemolytic activity by VvhA was measured using 1% human RBC suspension and the proteolytic activity of VvpE was determined by measuring caseinolytic activity. Iron stimulated the growth of MO6-24/O strain, and significantly stimulated the production of VvhA and VvpE (Figure 7). These results indicate that iron enhances the virulence expression as well as the growth of *V. vulnificus*, conversely, that iron limitation can downregulate the virulence expression as well as the growth of *V. vulnificus*.

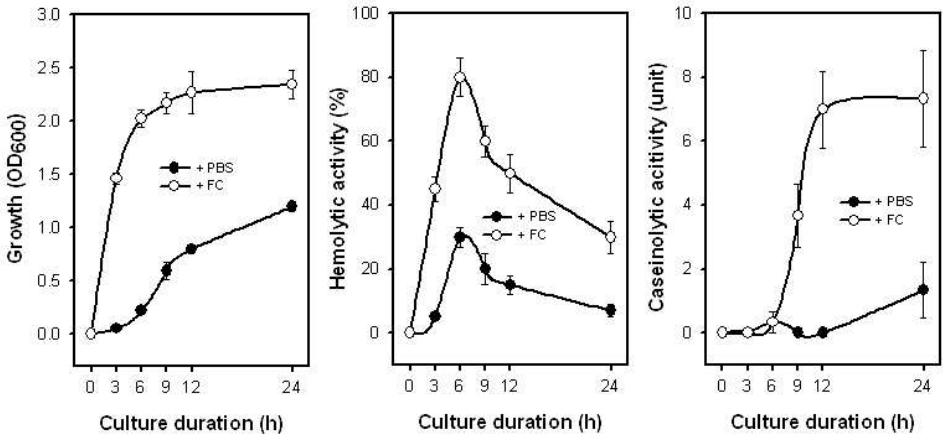


Figure 7. Effect of iron on the hemolysin/cytolysin (VvhA) and metalloprotease (VvpE) of *Vibrio vulnificus*. *V. vulnificus* MO6-24/O strain, grown in HI broth containing 100 μM dipyridyl, was inoculated in DF-HI or in DF-HI containing 10 μM FC at about 1 x 10^6 cfu/ml and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. During culture, culture aliquots were obtained at appropriate times to measure bacterial growth (OD_{600}; A) and culture supernatants were obtained to observe the productions of VvhA (B) and VvpE (C). The activity of VvhA was measured using 1% human RBC suspension and the activity of VvpE was determined by measuring caseinolytic activity, as described in the section of materials and methods. All assays were performed in triplicate and the results were expressed as means ± standard errors.

3.7. Ferriprox, but not Desferal, can inhibit the growth of *V. vulnificus*

In order to evaluate the potential usefulness of iron-chelators for the prevention or treatment of *V. vulnificus* infections, we inoculated *V. vulnificus* MO6-24/O strain into in DF-HI broth containing 10 μM FC and various concentrations of Ferriprox or Desferal (~150 μM) at 1 x 10^3 cfu/ml, and cultured with vigorous shaking (220 rpm) at 37^{o}C for 6 h. The growth of MO6-24/O strain was stimulated dose-dependently by Desferal, but inhibited dose-dependently by Desferal (Figure 8). Similar results were observed when the initial bacterial density was increased to 1 x 10^6 cfu/ml (data not shown). The growths of *S. aureus* and *E. coli* were also inhibited by Ferriprox, but at far higher concentrations (details will be reported elsewhere). These results indicate that Ferriprox, but not Desferal, inhibits the growth of *V. vulnificus* at low concentrations.

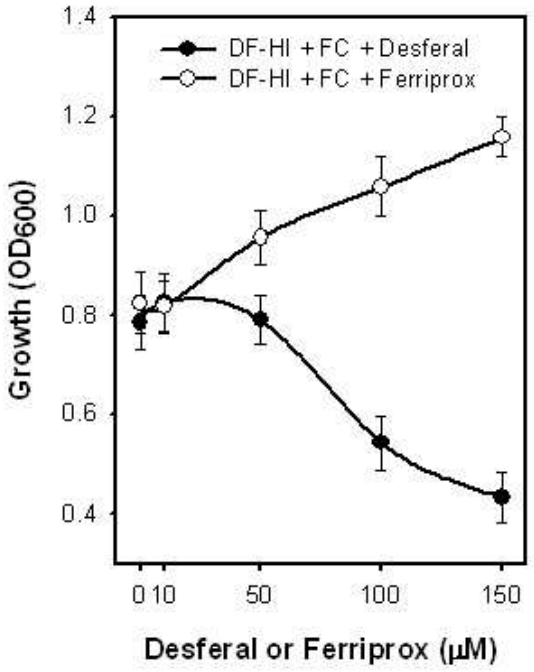


Figure 8. Effect of Ferriprox and Desferal on the growth of *V. vulnificus*. *V. vulnificus* MO6-24/O strain, grown in HI broth containing 100 μM dipyridyl, was inoculated into deferrated HI broth containing 10 μM ferric chloride (FC) plus Ferriprox or Desferal (~150 μM) at 1 x 10^3 cfu/ml, and cultured with vigorous shaking at 37°C for 6 h. Bacterial growths were monitored by measuring the OD_{600} values of culture aliquots. Results are expressed as the means and standard errors of OD_{600} values measured in triplicate.

3.8. The *desA* gene is expressed only in the presence of Desferal under iron-limited conditions

In order to determine expression of the *desA* gene, *V. vulnificus* MO6-24/O strain was inoculated into HI broths containing PBS or 200 μM DP plus 10~100 μM Desferal, DF-HI broths containing PBS or 50 μM Desferal and CA containing PBS or 50 μM Desferal at 1 x 10^6 cfu/ml, and cultured with vigorous shaking at 37°C for 6 h. Bacterial pellets were obtained to isolate total RNA and the amounts of total RNA were adjusted to 200 ng. RT-PCR was performed using the *desA*-specific primer set (DesA-3 and 4 targeting 355 bp), *vuuA*-specific primer set (VuuA-1 and 2; targeting 498 bp) and 16s rRNA-specific primer set (16s rRNA-F and R; targeting 211 bp). The expression of the *desA* gene was observed in HI broths containing DP plus Desferal in a proportion to the concentration of Desferal (Figure 9), but was not observed in HI broths containing PBS or only DP. Moreover, the expression of the *desA* gene was observed in DF-HI broth containing Desferal, but not in DF-HI broth containing only PBS. Similarly, the expression of the *desA* gene was observed in CA containing Desferal, but not in CA containing only PBS. In contrast, the *vuuA* gene encoding vulnibactin-receptor protein was expressed at higher levels in HI broth containing DP, in DF-HI broth and in CA than in HI broth containing PBS and regardless of the presence of Desferal, indicating that the expression of the *vuuA* gene is regulated by only iron-availability. These results indicate that the *desA* gene is expressed only in the presence of Desferal under iron-limited conditions.

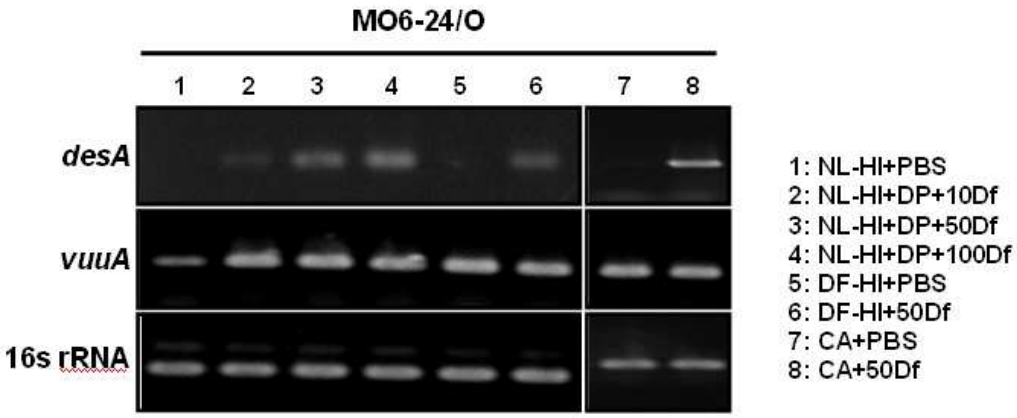


Figure 9. Expression of the *desA* gene in *V. vulnificus*. *V. vulnificus* MO6-24/O strain, grown in HI broth containing 100 μM dipyridyl, was inoculated into normal (NL) HI broth containing PBS or 200 μM dipyridyl (DP) plus 10~100 μM Desferal (Df), deferrated (DF) HI broth containing PBS or 50 μM Df and cirrhotic ascites (CA) containing PBS or 50 μM Df at 1 x 10^6 cfu/ml, and cultured with vigorous shaking at 37^{o}C for 6 h. Total RNA was isolated and RT-PCR was performed using the *desA*-specific primer set (DesA-3 and 4; 355 bp), *vuuA*-specific primer set (VuuA-1 and 2; 498 bp) and 16s rRNA-specific primer set (16s rRNA-F and R; 211 bp) as described in the section of materials and methods. Amplified products were electrophoresed on agarose gels and stained with ethidium bromide.

3.8. Mutation of the *desA* gene abolishes the response of *V. vulnificus* to Desferal

In order to confirm that DesA is the real Desferal-receptor in accordance with the molecular version of Koch's postulates [Falkow, 2004], we constructed a *desA*-deletion mutant (RC102) from the wild type MO6-24/O strain and complemented the wild type *desA* gene in the RC102 strain (resulting RC106 strain). The three strains were inoculated in DF-HI broths containing PBS or 50 μM Desferal at about 1 x 10^3 cfu/ml. The growth of RC102 strain was not stimulated in DF-HI broth regardless of the presence of Desferal (Figure 10). In contrast, the growth of RC106 strain was stimulated in DF-HI broth by Desferal. The growth of MO6-24/O was also stimulated by Desferal. Similar results were observed when PT was used as an iron source. These results indicated that mutation of the *desA* gene encoding DesA abolishes the response of *V. vulnificus* to Desferal, and thus DesA is the real Desferal-receptor fulfilling the molecular version of Koch's postulates.

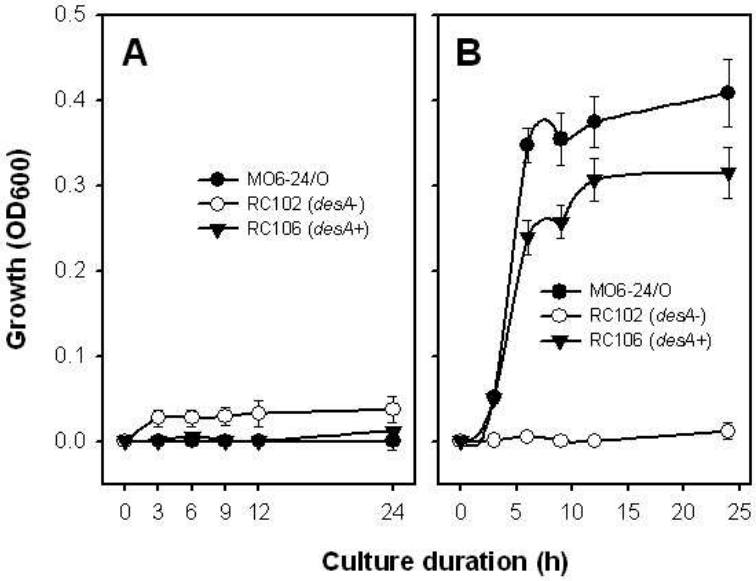


Figure 10. Effect of *desA* mutation on the growth of *V. vulnificus* in the presence of Desferal. The three strains, grown in HI broth containing 100 μM dipyridyl, were inoculated in deferrated HI broths containing PBS or 50 μM Desferal at about 1 x 10^3 cfu/ml, and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. During culture, culture aliquots were obtained at the indicated times to monitor bacterial growth. Bacterial growth was measured by the OD_{600} values of culture aliquots. Results are expressed as the means and standard errors of OD_{600} values measured in triplicate.

3.8. Ubiquitousness of the *desA* gene in *V. vulnificus* clinical and environmental strains

To determine that the *desA* gene encoding DesA is ubiquitously present in *V. vulnificus* clinical (n=10) and environmental (n=10) isolates, we performed PCR using the *desA*-specific primer set (DesA-1 and 2 targeting 863 bp). All strains were cultured in HI broths for 6 h. Culture aliquots were boiled using a microwave oven, and supernatants containing genomic DNA were obtained after removing cell debris by centrifugation at 13,000 rpm at 5 min. PCR was performed using the supernatants. The *desA*-specific band was observed in all *V. vulnificus* clinical isolates and in 9 of 10 environmental strains (Figure 11). Only one environmental isolate (E10 strain) were *desA*-negative. These results indicate that the *desA* gene encoding DesA is ubiquitous in *V. vulnificus* environmental and clinical isolates.

To further observe that the growths of these clinical and environmental isolates are stimulated by Desferal in accordance with the presence of the *desA* gene, we cultured them in DF-HI broth and DF-HI broth containing 50 μM Desferal. The growths of all clinical isolates were stimulated by Desferal (Figure 12). The growths of 9 of 10 environmental isolates were stimulated by Desferal, but the growth of one *desA*-negative environmental E10 strain was inhibited by Desferal. These results also indicate that the *desA* gene encoding DesA is ubiquitous in *V. vulnificus* environmental and clinical isolates and DesA is a real Desferal-specific receptor.

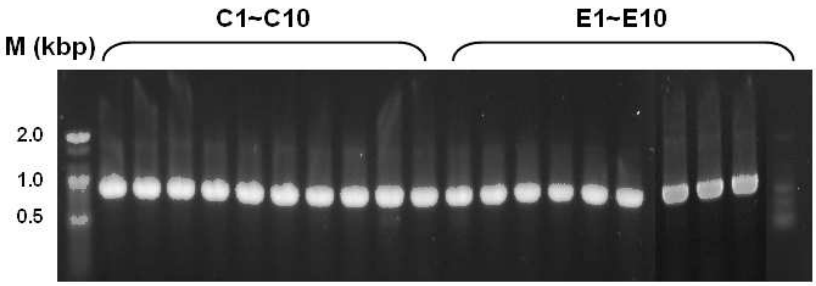


Figure 11. Ubiquitousness of the *desA* gene in *Vibrio vulnificus* clinical (C1~C10) and environmental (E1~E10) isolates. The *V. vulnificus* strains were inoculated into normal HI broths at 1 x 10^6 cfu/ml, and cultured with vigorous shaking at 37^{o}C for 6 h. Culture aliquots were mixed with lysis buffer, boiled for 10 min, and centrifuged at 13,000 rpm for 5 min to remove cell debris and to obtain supernatants containing genomic DNA. PCR was performed using the DesA-1 and 2 primer set, as described in the section of materials and methods. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. M: DNA size marker. The *desA*-specific band (863 bp) was observed in the 19 strains but not in the E10 strain.

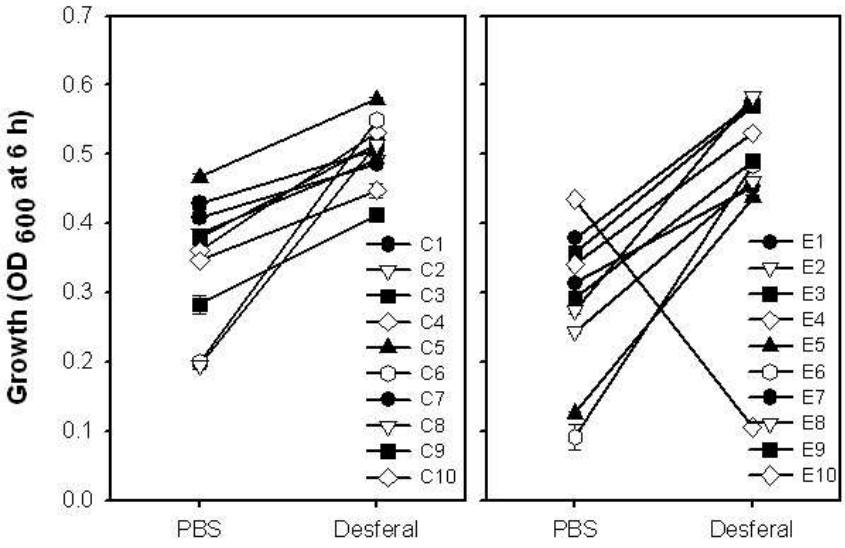


Figure 12. The growth stimulation of *Vibrio vulnificus* clinical (n=10) and environmental (n=10) isolates by Desferal. The *V. vulnificus* strains, grown in HI broth containing 100 μM dipyridyl, was inoculated into deferrated HI broth containing PBS or 50 μM Desferal at 1 x 10^6 cfu/ml, and cultured with vigorous shaking at 37°C for 6 h. Bacterial growths were measured the OD_{600} values of culture aliquots. Results were expressed as the means ± standard errors of OD values measured in triplicate. The growth of all the strains were significantly stimulated or inhibited by Desferal ($p<0.05$, Student-*t* test).

3.9. Ferriprox can inhibit the growths of all *V. vulnificus* clinical and environmental strains

To observe that the growths of clinical and environmental isolates were stimulated by Ferriprox regardless of the presence of the *desA* gene, we cultured them in DF-HI broth and DF-HI broth containing 150 μM Ferriprox. The growths of all clinical and environmental isolates were significantly inhibited by Ferriprox (Figure 13). These results indicate that Ferriprox can efficiently inhibit the *in vitro* growth of *V. vulnificus*, and suggest a possibility that Ferriprox therapy can be a useful means of preventing the *in vivo* growth of *V. vulnificus* in patients with iron-overload.

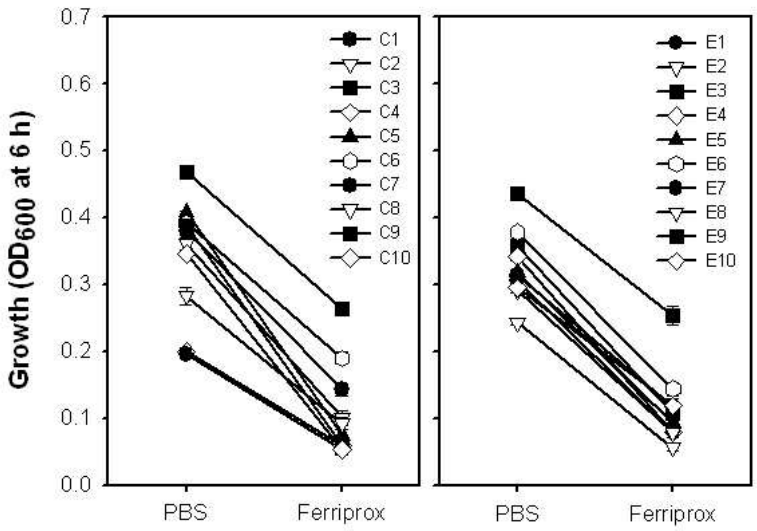


Figure 13. The growth inhibition of *Vibrio vulnificus* clinical and environmental isolates by Ferriprox. The *V. vulnificus* strains, grown in HI broth containing 100 μM dipyridyl, was inoculated into deferrated HI broth containing PBS or 50 μM Ferriprox at 1 x 10^6 cfu/ml, and cultured with vigorous shaking at 37^0C for 6 h. Bacterial growths were measured the OD_{600} values of culture aliquots. Results were expressed as the means ± standard errors of OD values measured in triplicate. The growth of all the strains were significantly inhibited by Desferal ($p<0.05$, Student-*t* test).

4. Discussion

The present study showed that TIC and IS were higher than normal in the majority of VVS patients (Figure 1) and thus that elevated serum iron is an important predisposing factor determining host susceptibility to *V. vulnificus*. Several lines of evidence support the notion that iron plays a pivotal role in the pathogenesis of VVS [Wright *et al*., 1981; Bullen *et al*., 1991; Brennt *et al*., 1998; Hor *et al*., 1998; Starks *et al*., 2000]. When mice were co-administered *V. vulnificus* and iron, the LD_{50} was lower than when iron was not co-administered [Wright *et al*., 1981; Starks *et al*., 2000]. Moreover, *V. vulnificus* is usually killed by normal human sera, but it grows rapidly in the sera of hemochromatosis patients or when the IS level of transferrin is intentionally raised to over 50% [Bullen *et al*., 1991; Brennt *et al*., 1998; Hor *et al*., 1998]. It is likely that an increase in IS lowers the antimicrobial activity of transferrin, and eventually increases host susceptibility to *V. vulnificus* [Neilands, 1991; Weinberg & Weinberg, 1995; Bullen *et al*., 2005].

However, increased host susceptibility to *V. vulnificus* cannot be explained by an increase in TIC or IS only. According to our results, more than 20% of VVS patients had TIC and IS levels in the normal range (Figure 1), and TIBC was not decreased although transferrin concentrations were lower than normal in 94% of VVS patients (Figure 2). These findings suggested that a portion of serum iron might be bound to other proteins besides transferrin in VVS patients. However, we could not directly measure such NTBI levels and *V. vulnificus* growth in VVS sera because the amount of the sera was insufficient. Hor *et al*. [1998] reported

that ferritin levels are increased due to release from damaged hepatocytes in patients with hepatic disorders such as liver cirrhosis and alcoholic hepatitis, which are well-known underlying diseases highly susceptible to *V. vulnificus.* Moreover, they also reported that ferritin levels rather than IS levels of transferrin are associated with the survival of *V. vulnificus* in whole blood. Helms *et al*. [1984] reported that phenylhydrazine-induced hemglobinemia increases mouse-lethality to *V. vulnificus*. *V. vulnificus* has been known to utilize NTBI such as ferritin and hemoglobin, and even hemoglobin-haptoglobin complex, as an iron source for growth [Helms *et al*., 1984; Simpson & Oliver, 1987; Zakaria-Meehan, 1988; Fouz *et al*., 1996; Litwin *et al*., 1996]. These findings indicate that NTBI, although present at very low levels, may play a more crucial role in determining host susceptibility to *V. vulnificus* than an elevated IS of transferrin. Accordingly, the presence of NTBI is likely to increase host susceptibility to *V. vulnificus* even in normal TIC or IS level settings.

V. vulnificus is believed to be a ferrophilic bacterium which requires a higher level of easily-available iron for growth initiation than other pathogens although *V. vulnificus* is several iron-uptake systems [Simpson & Oliver, 1983; Litwin *et al*., 1996; Webster & Litwin, 2000; Takata *et al*., 2005; Tanabe *et al*., 2005]. According to our results, serum iron levels were elevated in the majority of VVS patients (Figure 1) and *V. vulnificus* could acquire iron only from HT (Figures 4 and 6) but not PT. In addition, the growth of *V. vulnificus* was inhibited in media containing far smaller amounts of DP and AT than those required to inhibit the growths of *E. coli* and *S. aureus* (Figure 3). All these results indicate that *V. vulnificus* requires higher levels of readily-available iron for growth initiation than *E.*

coli and *S. aureus*. The reason why *V. vulnificus* is ferrophilic is believed to be because it produces smaller amounts of siderophores than *E. coli* and *S. aureus* (Figures 4 and 5). Accordingly, this ferrophilic characteristic of *V. vulnificus* is considered as the primary reason why *V. vulnificus* preferentially causes fatal septicemia in patients with elevated serum iron levels.

V. vulnificus is believed to be a ferrophilic bacterium which preferentially utilizes NTBI rather than TBI for growth initiation. According to our study, *V. vulnificus* could utilize TBI, but only at the relatively high initial bacterial density of 1×10^6 cfu/ml, and it preferred NTBI to TBI for growth initiation at the relatively lower initial bacterial density of 1×10^3 cfu/ml (Figure 6). In other studies [Litwin *et al*., 1996; Webster & Litwin, 2000], the growth of mutant strains incapable of producing vulnibactin or expressing vulnibactin receptor (called VuuA) has also been found to be stimulated by NTBI, but not TBI. These findings indicate that *V. vulnificus* prefers NTBI to TBI for growth initiation, especially at the low initial bacterial density which is likely to be better represent densities during the early stages of human infections. *S. epidermidis* is also known as a ferrophilic bacterium and a major causative microorganism of septicemia in neutropenic patients receiving intensive immunosuppressive or chemotherapy, which elevates serum total iron levels [Matinaho *et al*., 2001]. *S. epidermidis* is known to require higher levels of free iron for growth initiation and to utilize TBI less efficiently than *S. aureus* [Lindsay & Riley, 1994; Lindsay *et al*., 1995; Matinaho *et al*., 2001; von Bonsdorff *et al*., 2003; Park *et al*., 2005]. In addition, the ability of *S. epidermidis* to utilize TBI or NTBI is dependent on initial bacterial densities. Matinaho *et al*. [2001]

reported that *S. epidermidis* can utilize TBI only at high initial bacterial densities (over 1 x 10^6 cfu/ml), and that it prefers NTBI to TBI at the lower bacterial densities (approximately 1 x 10^3 cfu/ml) that reflect those during the early stages of human infections. Interestingly, despite these ferrophilic characteristics, *S. epidermidis* utilized TBI more efficiently than *V. vulnificus*; that is to say, *V. vulnificus* appears to be more ferrophilic than *S. epidermidis*. In our previous study, *S. epidermidis* could efficiently utilize TBI at the initial bacterial density of 1 x 10^6 cfu/ml [Park *et al*., 2005]. In contrast, *V. vulnificus* could not efficiently utilize TBI at the same density (Figure 3, 4, 5 and 6). Accordingly, this preference of *V. vulnificus* for NTBI is considered as one of the reasons why *V. vulnificus* can cause fatal septicemia even in patients with normal TIC or IS levels.

In addition, the cell density-dependent TBI utilization of *V. vulnificus* shown in the present study suggests that *V. vulnificus* iron-uptake systems, especially vulnibactin-mediated iron-uptake system, are under the control of the LuxS-quorum sensing system. The LuxS-quorum sensing system regulates the expression of *V. vulnificus* virulence factors in a cell density-dependent manner [Kim *et al*., 2003]. In *Actinobacillus actinomycetemcomitans*, mutation of the *luxS* gene encoding an enzyme for the synthesis of quorum-sensing signal molecule (called autoinducer 2) is known to regulate the expressions of genes involved in the transport and storage of iron [Fong *et al*., 2003]. However, no reports are available on the relationship between the iron-uptake system and the quorum-sensing system in *V. vulnificus,* and thus further studies on this relationship are necessary.

It is known that *V. vulnificus* also produces hydroxamate side-

rophore in addition to vulnibactin [Simpson & Oliver, 1988]. Hydroxamate siderophore is also able to facilitate *V. vulnificus* growth under iron-limited conditions, but vulnibactin is known to play a more important role in TBI utilization and in the virulence of *V. vulnificus* than hydroxamate siderophore [Litwin *et al*., 1996; Webster & Litwin, 2000]. However, in this study, *V. vulnificus* preferred NTBI to TBI for growth initiation at the relatively lower initial bacterial density of 1 x 10^3 cfu/ml (Figure 6). This finding suggests that the hydroxamate siderophore-mediated iron-uptake system may play a more important role in the growth initiation of *V. vulnificus* than the vulnibactin-mediated iron-uptake system. In *E. coli*, aerobactin (a hydroxamate siderophore) is known to be produced for the utilization of NTBI (or cell-driven iron), and enterochelin (a catechol siderophore) to be produced for the utilization of TBI. Aerobactin is known to be essentially required for *in vivo* growth because it is repeatedly reused and its activity is not reduced even in the presence of human serum, although it has far a lower affinity for iron than enterochelin [Williams *et al*., 1986; Brock *et al*., 1991]. However, the genes related to the hydroxamate siderophore-mediated iron-uptake system and the chemical structure of the hydroxamate siderophore have not been determined yet in *V. vulnificus*. Recently, a IutA-mediated iron-uptake system capable of utilizing *E. coli* aerobactin was identified in *V. vulnificus* [Tanabe *et al*., 2005]. However, it remains to be clarified whether or not *V. vulnificus* can utilize its own hydroxamate siderophore via this iron-uptake system, and thus further studies are necessary.

According to our results, iron stimulated the production of VvhA (Figure 7), which is known to be one of the most potent exotoxins pro-

duced by *V. vulnificus*. Production of VvhA is known to be regulated by several environmental factors [Paranjpye *et al*., 1998; Bang *et al*., 1999; Lee *et al*., 2000; Choi *et al*., 2002; Kim *et al*., 2003]. However, no reports have been available with regard to the relationship between iron and VvhA production although elevated serum iron is the most reliable factor determining host susceptibility to *V. vulnificus* and VvhA is one of the most potent exotoxins produced by *V. vulnificus*. In most bacteria, the expression of iron-regulated genes is generally negatively regulated at a transcriptional level by a ferric uptake regulator (called Fur) [Litwin & Calderwood, 1993]. Interestingly, it was reported that the *vvhA* gene has a putative Fur binding site in its regulatory region [Wright & Morris, 1991]. In spite of the presence of the Fur binding site, VvhA is more profoundly produced under iron-sufficient conditions than under iron-deficient conditions. This controversy suggests that *vvhA* transcription and extracellular VvhA production may be differently regulated by iron.

For long time, serious doubts have been raised about the pathogenic significance of VvhA since mutation of the *vvhA* gene was reported to have no effect on the lethality of mouse to *V. vulnificus* [Wright & Morris, 1991; Jeong *et al*., 2000; Fan *et al*., 2001]. Nevertheless, VvhA is still believed to be one of the virulence factors responsible for the pathophysiological changes observed in VVS although it is not the decisive factor determining mouse lethality. VvhA, at low levels, induces vasodilation and hemolysis or cytolysis [Kook *et al*., 1996 & 1999; Lee *et al*., 2004], stimulates the production of inflammatory cytokines including TNF-α [Park *et al*., 1996 & 1998], and induces superoxide anion-mediated apoptosis in human vascular endothelial cells [Kwon *et al*., 2001]. Moreover, VvhA is

evidently produced although its level is very low or its activity is easily inactivated in the presence of human body fluids [Lee *et al*., 2004; Choi *et al*., 2006]. Our results give a new suggestion that iron exacerbates the pathophysiological changes by facilitating the production of VvhA.

According to our results, iron stimulated the production of VvpE (Figure 7), which is known to be one of the most potent exotoxins produced by *V. vulnificus*. Purified VvpE has been shown to induce hemorrhagic damage and dermonecrosis, enhance vascular permeability and edema, and has also proven lethal to mice [Kothary & Kreger, 1987; Molla *et al*., 1989; Miyoshi & Shinoda, 1988 & 1992; Maruo *et al*., 1998]. However, as in the case of VvhA, serious doubts have been raised with regard to the roles of VvpE in the pathogenesis of VVS. VvpE-deficient mutants exhibited comparable virulence than were seen in the wild-type strains in studies employing mouse experimental models [Jeong *et al*., 2000; Shao & Hor, 2000; Fan *et al*., 2001]. In addition, our recent studies have indicated that VvpE exerts no direct effects on iron-assimilation from human transferrin [Shin *et al*., 2005] or on the inactivation of the hemolysin from *V. vulnificus* [Shin *et al*., 2005]. Nevertheless, from an evolutionary perspective, we theorize that VvpE is generated because it is essential for the survival of *V. vulnificus* in external environments as well as within human hosts. Accordingly, new paradigms will be required in order to elucidate the currently obscure pathogenetic roles of VvpE. Our results give a new suggestion that iron exacerbates the pathophysiological changes by facilitating the production of VvpE.

According to our results, the growth of *V. vulnificus* was completely suppressed by DP regardless of initial bacterial density despite the pres-

ence of HT (Figure 6) and inhibited by lower concentrations of DP or AT (Figure 3). Moreover, Ferriprox, but not Desferal, actually inhibited the growth of *V. vulnificus* clinical and environmental strains (Figure 8 and 13). These findings strongly suggest that iron-chelation therapy using Ferriprox can be used to prevent or treat *V. vulnificus* infections in susceptible patients.

Desferal is a kind of the streptomyces-driven hydroxamate siderophores and has been widely used as the standard iron chelating agent for the treatment of patients with iron overload. In addition, it has been reported that Desferal can inhibit the growth of ferrophilic *S. epidermidis* by lowering NTBI [van Asbeck, *et al*., 1983; Hartzen *et al*., 1988, 1991 & 1994]. However, this drug has been known to have some serious side effects, one of which is that this drug can stimulate the growths of some bacteria including *Y. enterocolitica* and *S. aureus.* These bacteria can use Desferal for efficient iron-uptake via specific receptors [Sebulsky *et al*., 2000; Lesic *et al*., 2002; Takata *et al*., 2005]. According to our results, the *desA* gene was expressed only in the presence of Desferal and found in all *V. vulnificus* clinical and environmental isolates. The growth of the *desA*-deletion mutant and *desA*-deficient spontaneous mutant were not stimulated in spite of the presence of Desferal (Figure 8, 9, 10, 11 and 12). Like these, some bacteria can utilize heterologous siderophores produced by other bacterial or fungal species. This strategy of called ‘siderophore piracy’ may be highly advantageous to such bacteria. The presence of this system implies a finite possibility of encountering the corresponding siderophores in environments with mixed bacterial populations such as the large intestine of human. Ferrioxamine E and G, structural

homologs of Desferal, have been known to be produced by *Erwinia* and *Hafnia* species in *Enterobacteriaceae* [Berner *et al*., 1988; Reissbrod *et al*., 1990]. Accordingly, it is not impossible that Desferal homologs secreted by members of gut commensal flora under some specific conditions may promote survival and proliferation of *V. vulnificus*. In addition, on the basis of our results, Desferal therapy in patients with iron-overload may rather increase the risk for VVS. These problems limit Desferal to be extensively used for the treatment of iron-overload.

In contrast, Ferriprox is a newly-developed synthetic oral iron chelator whose chemical structure is completely different from that of Desferal [Modell *et al*., 2000; Lesic *et al*., 2002]. Because of this structural difference, it is likely that Ferriprox, but not Desferal, inhibit the growth of *V. vulnificus*. Accordingly, Ferriprox appears to be a useful means for the treatment or prevention of *V. vulnificus* infections by reducing both the levels of NTBI and TBI in patients with iron-overload. The removal of NTBI *in vitro* and *in vivo* by Ferriprox is known to be usually faster than TBI [Kontoghiorghes, 2006]. It was reported that Ferriprox could also inhibit the growth of *Y. enterocolitica* by decreasing iron-availability [Lesic *et al*., 2002]. In addition, we recently found that Ferriprox could inhibit the growth of staphylococci including methicillin-resistant *S. aureus* [details will be reported elsewhere]. Moreover, to data, there is no report on bacteria capable of utilizing Ferriprox for iron uptake.

5. Conclusion

V. vulnificus preferentially causes fatal septicemia in iron-overloaded patients because of its ferrophilic nature, and *V. vulnificus* can cause a fatal septicemia even in a normal TIC or IS level setting because of its preference for NTBI. Iron stimulates the expression of *V. vulnificus* virulence by facilitating the production of hemolysin/cytolysin and metalloprotease. In addition, the present results suggest that iron-chelation therapy using Ferriprox, but not Desferal, can be an effective means of preventing and treating *V. vulnificus* infections by reducing NTBI and TBI levels in susceptible patients.

6. References

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Abstract

Potential Usefulness of Iron-chelating Therapy for the Prevention of *Vibrio vulnificus* Septicemia

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Background and objective) *Vibrio vulnificus* is a gram-negative halophilic bacterium and known to cause fatal septicemia in patients with elevated serum or tissue iron levels although it possesses several iron-uptake systems. Accordingly, we tried to directly measure serum iron levels and transferrin levels in *V. vulnificus* septicemia (VVS) and to compare with those in normal healthy persons, to determine the ferrophilic characteristic of *V. vulnificus* and the effect of iron on the expression of *V. vulnificus* virulence factors, and then to evaluate the potential usefulness of iron-chelation therapy for the prevention or treatment of VVS.

Methods and results) We directly measured serum iron levels and transferrin levels in VVS and the levels were compared with those in normal healthy persons. Serum total iron concentrations and transferrin iron saturations were significantly elevated in VVS patients (n=33) versus normal controls (n=45) ($p<0.05$). Transferrin levels were significantly elevated in VVS patients versus normal controls ($p<0.05$). We determined the ferrophilic characteristic of *V. vulnificus*. *V. vulnificus* preferred non-trans-

ferrin-bound-iron (NTBI) to transferrin-bound-iron (TBI) for growth initiation. In addition, *V. vulnificus* required higher levels of available NTBI for growth initiation, produced siderophores at smaller levels and utilized TBI less efficiently than *Staphylococcus aureus* or *Escherichia coli.* Moreover, *V. vulnificus* could not utilize TBI for growth initiation at an initial bacterial density of about 1 x 10^3 cfu/ml, which is a reasonable bacterial density in human *V. vulnificus* infections. Rather, NTBI was required for the efficient TBI utilization by and the growth initiation of *V. vulnificus*. We determined the effect of iron on the expression of *V. vulnificus* virulence factors. Iron stimulated the production of *V. vulnificus* cytolysin/hemolysin and metalloprotease. We evaluated the potential usefulness of iron-chelation therapy for the prevention or treatment of VVS. The growth of *V. vulnificus* was inhibited dose-dependently by Ferriprox®, a new clinically available oral iron chelating agent, but rather stimulated dose-dependently by Desferal®, which has been used as the standard iron chelating agent. The *desA* gene, which encodes for 78 kDa outer membrane protein (DesA) which can bind to Desferal, was present in 10 of 10 clinical strains and in 9 of 10 environmental strains. The growths of all the *desA*-positive strains were stimulated by Desferal, but the growth of one *desA*-negative environmental strain was rather inhibited by Desferal. The transcription of the *desA* gene was observed only in the presence of Desferal under iron-deficient conditions, but not in the absence of Desferal under iron-deficient conditions or under iron-sufficient conditions. A *desA*-deletion mutant did not grow despite the presence of Desferal under iron-deficient conditions, but its suppressed growth was completely recovered by the *in trans* complementation of wild-type *desA* gene. In contrast with Desferal,

Ferriprox inhibited the growths of all *V. vulnificus* clinical and environmental strains regardless of their abilities to utilize Desferal.

Conclusion and suggestion) These results show that *V. vulnificus* is a ferrophilic bacterium that requires higher levels of available NTBI than other pathogens, prefers NTBI to TBI for growth initiation, and thus iron-chelation therapy using Ferriprox can be a useful means of preventing VVS by reducing iron-availability in susceptible patients. In addition, these results indicate that DesA is the real Desferal-specific receptor expressed only in the presence of Desferal under iron-deficient conditions and ubiquitously present in *V. vulnificus* clinical and environmental strains, suggesting that Desferal therapy to improve iron overload is rather likely to predispose fatal VVS.

Key words: *Vibrio vulnificus*, Ferrophilic bacterium, Iron, Iron-chelation, Desferal (Deferoxamine), Ferriprox (Deferiprone)

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다. 이들의 따뜻한 관심과 끊임 없는 애정은 지치고 힘들 때마다 저에게 큰 힘이 되어 주었습니다.

부족한 오빠를 대신하여 장녀 역할을 너무나 듬직하게 잘해준 동생 혜진이에게 고마움을 전합니다. 공부한다는 핑계로 가족에게 소홀했던 이 못난 오빠에게는 항상 든든하고 믿음직스러운 동생이었습니다. 어서 빨리 행복한 가정을 꾸미길 기원합니다.

마지막으로, 제모든 힘의 원천이며 살아가는 원동력인 사랑하는 부모님께 감사드립니다. 두분의 헤아릴 수 없이 가득한 사랑과 관심, 인내가 없었다면 지금의 저는 결코 있을 수 없었을 것입니다. 그 무엇으로도 다 표현할 수 없을 만큼의 헌신적인 두 분의 사랑으로 이 자그마한 논문은 빚어졌습니다. 두분의 사랑에 항상 감사하며 최선을 다해 열심히 살아가겠습니다.

이 외에 제가 미처 언급하지 못한 고마운 분들이 너무나 많습니다. 그 분들의 이름을 하나 하나 되새기지 못함을 죄송하게 생각하며, 대신 제 깊은 감사의 말로 이 글을 마칠까 합니다. "모두들 감사드립니다."

2006년 12월 동지에 즈음하여 백악동산에서

박 용 진

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논문제목	한글 : **비브리오 패혈증의 예방을 위한 철착화제의 유용성** 영어 : **Potential Usefulness of Iron-chelating Therapy for the Prevention of *Vibrio vulnificus* Septicemia**				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(o) 반대( )

2007 **년** *2* **월 일**

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