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Characterization of the Nature of Virulency Caused by *Vibrio anguillarum*: Identification of an outer Membrane Protein Rendering Virulency

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Virulent factors from two strains of a fish pathogenic microorganism, *Vibrio anguillarum*, V7 and V104, have been studied to identify the nature of virulency and mechanism of pathogenicity. The most virulent strain V7 (serotype J01) was compared for various characteristics with less virulent one V104 (serotype J03). The effect of temperature on the reduction of virulency of *V. anguillarum* was critical above the optimal temperature, regardless of the strains ever tested. The optimal temperature for virulency induction was 22~24°C. Dramatic decrease of 41 kd outer membrane protein in V7 grown at 32°C suggested the possibility that this protein might be associated with the virulency, whereas 31 kd one from V104 was not so significant in rendering virulence even if it was also reduced when grown at high temperature. Strain V7 showed serum resistance, hemagglutinating activity, and proteolytic activity. It is not clear yet if strains V7 and V104 have different mechanisms of pathogenicity, but it is likely that the virulency of the strain V7 could be directly related to the expression of 41 kd protein.

KEY WORDS □ ***Vibrio anguillarum*, vibriosis, outer membrane proteins, proteolytic activity**

Vibrio anguillarum has been known as an important pathogenic microorganism infecting fresh water fish (13, 19, 36), as well as marine fish (3, 18, 27, 33, 34), especially causing vibriosis in salmonoid (1, 15, 20). This disease can be characterized with organ hemorrhage and tissue necrosis with high mortality (32). Due to great economical loss in fish farming, pathogenic nature of *V. anguillarum* has been intensively studied on its genetic or growth factors controlling virulency during infection. The pathogenesis, however, is not still clearly disclosed providing only the basic data to understand how this pathogen develops virulency during infection.

Crosa *et al.* (12) reported the presence of specific plasmid (pJM1), 50 kbp in size, responsible for the virulency only in highly pathogenic strains of *V. anguillarum* such as NCMB0572, LS173, 775, ES-1, 133-S, RG 75834, and V1. Curing the plasmid from highly toxic *V. anguillarum* with incubation at high temperature was also known to induce virulency loss (11), implying some important roles of plasmid in developing virulency. While isogenic derivatives without plasmid could not grow in iron deficient environment, the fact *V. anguillarum* strain V104 with pJM1 could survive explains that pJM1 strongly stimulates activation of the efficient iron take-up devices (10).

In addition, Crosa *et al.* (12) also found that 86 kd outer membrane protein (OM2) could be detected from virulent strains. The outer membrane OM2 binding to 79 kd protein (OM3) was suggested to increase the efficiency of iron uptake. Purified pJM1, however, did not exert virulency when transformed into *E. coli* (personal communication with Prof. J.L. Fryer, Oregon State University), and it is reported to have no homology with other known plasmid such as pCOlV-K30, from *E. coli*, which has similar function in iron uptake (39). It is not likely that pJM1 is functionally related to virulency, as high virulency caused by hemolysis (28) or serum resistance (39) was reported not to be directly controlled by genes located in plasmid.

Exotoxin from *V. anguillarum* has been frequently reported by several groups. Heat labile exotoxin with proteolytic activity and heat stable one with hemolytic and proteolytic activity had been suggested as the virulent factors by Inamura *et al.* (22) from *V. anguillarum* ATCC 19264 or PT81049 (serotype J01), and by Kodama *et al.* (24)

from *V. anguillarum* NCMB1 (serotype J03). The presence and identification of exotoxin in these microorganisms are not confirmed yet (personal communication with Dr. R. Hedrick, U.C. Davis).

One of the studies on endotoxin as a possible virulent factor was reported by Harvell *et al.* (23). They injected isolated endotoxin into salmonoid fish finding no significant virulency developed, which implies that it is still obscure if endotoxin is related to vibriosis.

We have been studying on virulent factors of *V. anguillarum* V7 (NCCMB6 serotype J01) and V104 (NOAA V-775, serotype J03) focusing at possible roles of toxic molecules rendering proteolytic and hemolytic activities. We now report that presence and increased expression of an outer membrane protein were correlated with high virulency at least in the strain V7.

MATERIALS AND METHODS

Strains

Vibrio anguillarum strain V7 (NCMB6, serotype J01) and strain V104 (NOAA V-775, serotype J03) were kindly provided by Prof. T. Kimura (Hokkaido University) in 1988 and maintained in our laboratory thereafter. Highly virulent strains were passaged in tryptic soy agar (TSA, Difco) supplemented with 1% NaCl (Merck) at 22~24°C. Virulency was maintained by *in vivo* passage using carp fries, *Cyprinus carpio* L., and reisolates were confirmed by 0/129 test and TCBS media (7).

Determination of LD_{50}

V. anguillarum strains V7 and V104 were cultured in TSB supplemented with 1% NaCl for 24 hrs either at 23°C or at 32°C. Bacterial cells were harvested by centrifugation (5,000 rpm, 10 min, RC-5C Sorvall, SA600 rotor, Sorvall Instrument), washed three times in PBS, and resuspended in PBS for injection after adjusting to proper concentration which was confirmed afterwards by viable count.

Each group of four carp fries was injected with 0.1 m*l* bacterial suspension after anesthetization with ethyl-*p*-aminobenzoate (benzocaine, Sigma). Chemicals were purchased from Sigma unless otherwise mentioned. They were kept for a week at 20°C aquarium with continuously supplied dechlorinated tap-water. Death was scored to be caused by vibriosis only when confirmed by reisolation of *V. anguillarum*. LD_{50} was determined by the methods described by Reed and Muench (23).

Serum resistance

Serum resistance of *V. anguillarum* was determined by the methods of Munn *et al.* (29) and Trust *et al.* (38). Bacterial cells were cultured in TSB for 4~5 hrs so that they could be in exponential stage at the time of experiments, and washed three times in 0.15 M NaCl at the concentration of 10^7 cells/m*l*.

Total volume of 1.4 m*l* of rabbit antisera, prepared fresh, was mixed with 0.1 m*l* of bacterial suspension. Bacterial cultures incubated at 23°C were counted every hour by standard spread-plate method for viable number. Heated serum at 56°C as well as normal rabbit serum was also tested bactericidal activity in the same way.

Hemagglutination

Human blood type O RBCs were prepared from peripheral blood of normal healthy volunteers by Ficoll/Hypaque (p=1.077, Sigma). After washing three times in PBS (pH 7.4), RBCs were resuspended in PBS to make 3% (v/v) solution and kept at 4°C until use. Bacterial cells were adjusted to the concentration of 1.1×10^9 cells/m*l*, which was confirmed by hemacytometer and viable count. Strains V7 and V104, grown under the condition either to induce virulency or not to, at the above mentioned concentration, were added to the equal volume of RBC suspension on slide glasses (2). Hemagglutination was determined in 10 min with gentle stirring of the slide glasses. The minimal number of bacterial cells required for hemagglutination was also estimated by the same method explained.

Proteolytic activity

Proteolytic activity, as one of the suspected virulent factors, was measured by methods of Kreger and Griffin (25) and Kunitz (26) with slight modification. In brief, total volume of 0.5 m*l* casein solution (6% in 0.2 M Tris-Cl, pH 7.2 or 8.0) was mixed with 0.4 m*l* of bacterial suspension (5.0×10^8 cells/m*l*, confirmed by viable count), and kept at 25°C for 20 min. Reaction was stopped by addition of 3.5 m*l* of 5% trichloroacetic acid (TCA) solution. Precipitates were removed by centrifugation at $4,000\times g$ for 10 min. Total volume of 4.5 m*l* NaOH (0.5 M) was added to supernatant and proteolytic activity was evaluated by measuring absorbency at 275 nm using spectrophotometer (UV-160, Shimatsu).

Cell extract and cytopathic effects (CPE) on fish cell lines

Cell extract with sonication (Lab-Line System, Lab-Line Instruments) was filtered with nitro-cellulose filter (Sigma), and protein concentration was adjusted to 750 μg/m*l* after freeze-drying.

Lipopolysaccharide extraction was carried out according to the method of Wesphal and Jann (40). Bacterial cells weighing 1 g after freeze-drying were resuspended into 17.5 m*l* of distilled water preheated to 65~68°C. Equal volume of phenol at the same temperature was added and vigorously mixed. Aquatic layer was repeatedly collected by centrifugation at $3,000\times g$ for 30~45 min, and dialysed against distilled water for 3~4 days followed by freeze-drying. Each LPS preparation was resuspended in calcium-magnesium free PBS (CMF-PBS) and adjusted

to the concentration of 100 μg/ml after filtration through nitrocellulose membrane (0.2 μm, Nalgene) to make protein free cell extract. The same preparation was made by filtering through cellulose acetate membrane (0.2 μm, Pharmacia) to get the cell extract containing with protein components.

Cell extracts were tested for its virulency on established fish cell lines such as CHSE-214, EPS, and RTG-2, which were kindly provided by Prof. J.L. Fryer in 1987 and kept thereafter in our laboratory. Cells transferred to 96-multiwell plates were infected with serially diluted cellular extracts, and incubated at 20~22°C in the low temperature incubator (Sargent-Welch Scientific Co.) for 10 days and CPE development was scored.

Estimation of endotoxin and exotoxin

The concentration of bacterial protein was adjusted to 200 μg/ml after freeze drying as explained, and followed by filtration. Quantification of protein was performed by Bradford's method using Coomassie brilliant blue G-250.

Each preparation of concentrated cell-free culture supernatant or cells at appropriate concentration was injected into peritoneal cavity of goldfish to identify and evaluate the presence of exotoxin.

Analysis of outer membrane proteins

Outer membrane was isolated according to the method of Crosa and Hodges (18). Cells grown in 50 ml TSB supplemented with 1% NaCl were harvested and resuspended in 10 ml of 10 mM Tris-HCl supplemented with 0.3% NaCl (pH 8.0) for sonication. Supernatant from sonicated lysate was centrifuged at 13,000×*g* for 2 hrs to precipitate cellular envelope. Inner membrane was removed by the method of Filip *et al.* (17) immersing cellular envelope into 1.5% sodium lauryl sarcosinate (sarkosyl, w/v), and kept at R.T. for 30~40 min. Insoluble outer membrane was obtained by centrifugation at 13,000×*g* for 3 hrs. This was repeated at least twice for complete removal of inner membrane.

Outer membrane was lysed in lysis buffer prepared with 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 0.001% bromophenol blue, 5% beta-mercaptoethanol (Merck), and 1 mM phenyl methyl sulfonyl fluoride (PMSF). After boiling for 5 min, outer membrane proteins were analyzed on 12.5% SDS-polyacrylamide gel electrophoresis.

Isolation of plasmid

Plasmid was isolated by using Crosa's method (9). In brief, cells grown to 2×10^8 cells/ml in TSB were harvested and lysed on ice bath for 5 min in 0.1 ml lysis buffer prepared with 0.1 M Tris-HCl and 1 mM EDTA, and 25% sucrose (pH 8.0). The lysate was mixed with 0.5 ml of 20% SDS solution in TE buffer (0.05 M Tris-Cl, 0.02 M EDTA, pH 8.0) and stirred well. The reaction mixture was put into 55°C water bath for 15 sec and at room temperature for another 15 sec with gentle stirring, and this had been repeated for eight times to obtain very viscous extract. Alkaline denaturation was carried out with 0.5 ml of 3 N NaOH, and neutralized by gentle stirring with 1.0 ml of 2 M Tris-Cl (pH 7.0).

Precipitates obtained by the addition of 0.65 ml of 20% SDS and 1.25 ml of 5 M NaCl was resuspended in TE buffer. Plasmid was further purified by extraction once with phenol and twice with chloroform, and finally by ethanol precipitation. Plasmid DNA resuspended in TE buffer (pH 7.6) was analyzed on 0.7% agarose gel electrophoresis.

Table 1. *Toxicity[a] of Vibrio anguillarum strains V7 and V104.*

Strain	V7 (NCMB6, J01)		V104 (NOAA V-775, J03)	
Culture temperature	23°C	32°C	23°C	32°C
Mortality (%)[b]	100 (8/8)	75 (6/8)	50 (4/8)	12.5 (1/8)
Mean time to death (hr)	17	25.4	76	106
LD_{50}[c]	1.5×10^4	3.0×10^6	4.5×10^7	5.3×10^7

[a] Carps were injected intraperitoneally with 0.1 ml of 1.3×10^7 bacterial cells/ml in PBS, and maintained at 25°C for 7 days.
[b] Mortality was described by percentage of the number of dead carps to that of the tested.
[c] Mean of two separate experiments.

RESULTS

Evaluation of virulency

Virulency of strains V7 and V104 was first compared in mortality, expressed by the time from infection to death, and in LD_{50} titer (Table 1). In conclusion, it is likely that strain V7 was more virulent than V104, and the virulency was significantly influenced by culture temperature since the both strains were more virulent at 23°C than at 32°C The temperature effect on strain V 104, however, was much less obvious than on strain V7, as previously reported (7).

Serum resistance

Serum resistance of pathogenic microorganisms was suggested to explain how they could escape the attack of host immune system upon infection. Two strains of *V. anguillarum* were examined if they were resistant to humoral immunity of the host. In Table 2, susceptibility of the host to the infected microbial pathogens was expressed in survival rate after reaction with antisera.

As complement proteins were known to play important roles in microbial infection, rabbits, instead of fish, were immunized against two strains of *V. anguillarum* in order to have enough

Table 2. *Survivability[a] of V. anguillarum with serum resistance against the bactericidal activity of rabbit antisera.*

Antiserum	V7 (NCMB6, J01)		V104 (NOAA V-775, J03)	
	23°C	32°C	23°C	32°C
Anti-V7	N.S.[b]	8.8%	2.2%	0.9%
Anti-V104	N.S.	6.9%	0.9%	0.2%
Normal	N.S.	75.0%	0.4%	N.S.
Heated[c]	N.S.	N.S.	N.S.	N.S.

[a] Estimated by viable count on TSA plates.
[b] Not susceptible.
[c] Both immune and non-immune (normal) sera were boiled at 56°C for 30 min.

Table 3. *Hemagglutination activity[a] of V. anguillarum with human group O RBCs.*

Strain	Culture temperature	Minimum concentration of bacteria required for hemagglutination[b]
V7 (NCMB 6, J01)	23°C	5.88×10^{8} cells/ml
	32°C	N.D.[c]
V104 (NOAA V-775, J03)	23°C	1.18×10^{9} cells/ml
	32°C	N.D.

[a] Experiments performed with 3% human group O blood at room temperature.
[b] The minimum agglutinating dose was defined as the lowest number of bacteria per ml that showed visible agglutination within 10 min.
[c] Not detected even with 2×10^{9} cells/ml.

amount of effector molecules in sera. Strain V7 only could survive showing serum resistance when cultured at the optimal temperature, meanwhile V104 and V7 grown at 32°C lost the resistance. Since the loss of serum resistance was correlated with the virulence loss at the same time, virulency might be at least partly exerted by serum resistance.

Both strains were virulent when reacted with heated or even with normal rabbit sera, suggesting that immune reaction was activated to bacterial infection, and that bactericidal activity was rendered by alternative complement pathway. The resistance of strain V104 to antiserum was low, but increased upon addition of normal serum.

Hemagglutination induced by virulent *V. anguillarum*

Strains V7 and V104 were cultured at high temperature of 32°C resulting in much less virulency. Human blood type O RBCs were agglutinated only when bacterial cells were added (Table 3). The minimal concentration of bacterial cells per unit volume increased for hemagglutination upon the decrease of virulency. The difference, however, was not so significant, and it should rather be considered that the ability of hemagglutination is only a requirement but not an important factor for induction of virulency.

Table 4. *Analysis of strain V7 for cytopathic effect (CPE)[a] in fish cell lines.*

Sample		Ratio of CPE[b]
LPS[c]	*V. anguillarum* V7	0/8
	S. typhimurium	0/8
	E. coli	0/8
Bacterial cell extract	with proteins[d]	6/8
	without proteins[e]	0/8

[a] Samples were inoculated to multiwell plates where fish cells (EPS, CHSE-214, and RTG-2) were grown for 6 or 24 hrs, and PBS was used as a control.
[b] Ratio of number of wells showing CPE to number of wells inoculated.
[c] Inoculated amount of LPS was 0.1 ml of 500 μg/ml.
[d] Prepared with sonication and filtration using cellulose acetate membrane filter (pore size 0.2 μm, Pharmacia).
[e] Prepared with sonication and filtration using nicrocellulose membrane filter (pore size 0.2 μm, Nalgene).

Table 5. *Proteolytic activity of Vibrio anguillarum.*

Strain	Culture temperature	Proteolytic activity (A_{275})	
		pH 7.2	pH 8.0
V7 (NCMB 6, J01)	23°C	0.35[a]	0.51[b]
	32°C	N.D.[c]	N.D.
V104 (NOAA V-775, J03	23°C	0.03	0.33
	32°C	N.D.	N.D.

[a] 0.6% casein in Tris-HCl, pH 7.2.
[b] 0.6% casein in Tris-HCl, pH 8.0.
[c] Not detected.

Cytopathic effect (CPE) development and proteolytic activity

The virulent strain V7 induced CPE in fish cell culture. Table 4 summarizes that CPE was induced by protein molecules, and this strongly suggests the possibility of the presence of some proteolytic activities to exert virulency. Proteolysis could explain tissue necrosis and hemorrhage in peritoneal cavity, which are the main symptoms of vibriosis. As shown in Table 5, both strains were of very strong proteolytic activities, and no activity was detected when grown at 32°C.

Virulency and outer membrane proteins

Culture filtrate collected after cells were grown at the optimal temperature was estimated for the virulency by injecting into peritoneal cavity of goldfish. Virulency, however, could be detected

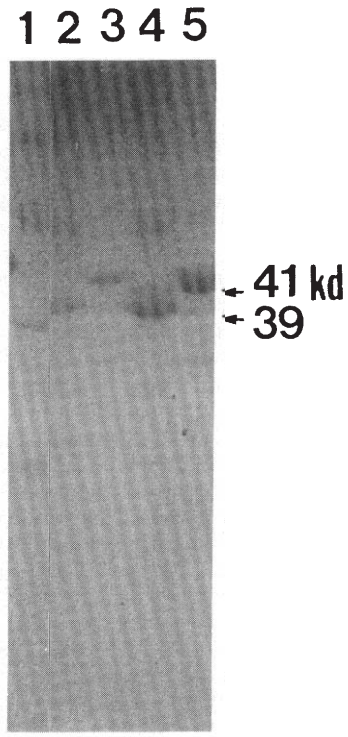

Fig. 1. *SDS-polyacrylamide gel electrophoretogram (12.5%) with outer membranes of Vibrio anguillarum grown at different temperatures.* Shown are small molecular weight markers (lane 1), outer membrane proteins from V7 and V104 grown at 32°C (lanes 2, 3) and grown at 23°C (lanes 4, 5) in order, respectively. Each lane was loaded with 50 μl of protein at the concentration of 200 μg /ml.

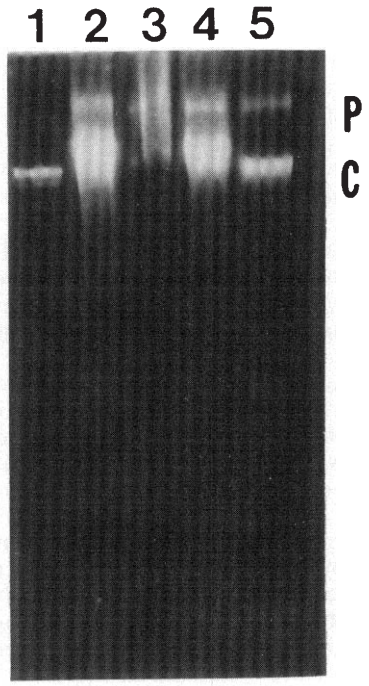

Fig. 2. *Plasmid from Vibrio anguillarum was identified on agarose gel electrophoretogram.* Shown are marker λ DNA fragments disgested with *Bst*II (lane 1), and plasmids from V7 grown at 23°C and 32°C (lanes 2, 3), and plasmids from V104 at the same temperatures (lanes 4, 5), respectively. C and P stand for chromosomal and plasmid DNA, respectively.

only when washed bacterial cells were administered (data not shown).

Outer membrane protein profiles are shown in Fig. 1. Expression of two different proteins, 41 kd in V7 and 39 kd in V104, was increased in amount when grown at the optimal temperature with high virulency. Even though the amount of 39 kd protein was significantly increased, the increase in virulency was not that much great. Increase of 41 kd protein, however, could be correlated with virulency, suggesting that it could be mainly related to the virulency.

Isolation of plasmid

A DNA band regarded as plasmid with approximate size of 80 kbp was detected on agarose gel electrophoresis from both strains and both cultures, at 23°C and at 32°C (Fig. 2). The amount of chromosomal DNA was also much thicker suggeting different growth rate at different temperature. The presence of plasmid, however, does not explain the different virulency of two strains.

DISCUSSION

To explain how pathogenic microorganisms exert pathogenicity in host animals, the possible roles of bacterial resistance to the attack of humoral immune responses, hemagglutination followed by hemolysis, and many other factors have been under intensive studies (21, 37, 38).

Even though the severe damage caused by vibriosis in fish farming had been reported earlier, the exact nature of vibriosis is not clear until now. The basic question whether it is exotoxin or endotoxin is in controversy, but most of early studies (3, 22, 24) supported the possibility of exotoxin.

Since it has been believed that highly virulent pathogens would stimulate the immune system very strongly, they should be equipped with some

mechanisms either to avoid or to overcome such attack. In homeotherm animals, complement proteins play important roles in inhibiting bacterial tissue destruction, regardless of specific immunity of the host (16). It is also known that fish have complement system (6), and it is inhibitory for bacterial infection (14, 31). The role in poikilotherm animals, however, is not clearly revealed.

We have used rabbit antiserum instead of fish only to secure enough amount of sera for experiments, and this was based on the study that rabbit serum has similar effect on fish-pathogenic bacterial growth and on serum resistance (35). It is likely that alternative pathway was activated, and the activation is more stimulated by bacterial infection (30). The serum resistance shown by strain V7 was in context with the assumption. Virulency of strain V104 was completely demolished by antibodies in immunized serum, which was not observed in normal serum.

Attachment of bacteria was regarded as the first step to develop tissue necrosis or hemolysis for RBCs, which was estimated by hemagglutination activity. In our separate report, it was shown that when vibrio culture was added to RBC solution, they did not seem to bind to the surface of human RBC (7). We recently found that, with the less virulent strains, more bacterial cells were required and it took longer until we could observe hemagglutination on slide glasses. The difference in virulency could be expressed by minimal concentration of hemagglutination induction. Nevertheless, hemagglutination by *V. anguillarum* seems only necessary but not essential to exert virulency.

From CPE observed in fish cell cultures, proteolytic activity appeared to be one of the possible mechanisms for infecting fish tissues in natural infection. The proteolytic activity found in *V. anguillarum* was fairly high when compared with other enzymes (25, 26).

In addition, we have tested hemolytic activity for one of the candidates to explain the virulency of *V. anguillarum*. Unfortunately we tested it using sheep RBCs, instead of human ones. As reported previously, sheep RBCs are hardly susceptible to vibrio hemolysin. Even though no relationship between hemolytic activity and virulency could be drawn from this experiment, the possibility that the protein responsible for virulency could be the molecule with hemolytic activity is now under study.

Two proteins even with slightly different size might be responsible for the pathogenicity of *V. anguillarum*. The 41 kd protein detected in strain V7 increased significantly when it was grown to induce virulency. Thus, it can be explained that the virulency is most probably brought about from 41 kd protein. The 39 kd one from strain V104, however, was reduced very much, at least in amount, as much as its virulency decreased. It still should be responsible for virulency of strain V104.

Since it has been known long that pathogenic microorganisms contain lipopolysaccharide and outer membrane proteins (4, 5, 8). When the virulency was changed according to temperature shift, LPS or outer membrane proteins expressed in different amount should be considered to be directly related to virulency. This study shows that the responsible molecules are proteins, implying outer membrane proteins. The function of the 41 kd protein, especially, is not clear yet, and it is necessary to isolate the protein in pure forms to examine its virulency.

Crosa *et al.* (9, 11, 12) have studied long on the plasmid isolated from virulent *V. anguillarum*. The plasmid, pJM1, is believed to encode siderophore sequestering iron from host, and this was only a device for survival since strains without plasmid are not virulent hence not being able to survive (10, 39). This viewpoint is still controversial as no correlation between virulency and the presence of plasmid was supported by Munn (28) and Trust *et al.* (39). We also studied the presence of plasmid in virulent strains of *V. anguillarum* finding 80 kbp plasmid from both strains V7 and V104, and it is suggested that virulency might not directly be rendered by gene products on this plasmid.

Iron sequestration was examined to see if the growth could be affected by the absence of the plasmid. When bathophenanthroline-disulfonic acid (4,7-diphenyl-1,10-phenanthroline-disulfonic acid) was added at the final concentration of 3 μM to medium as iron chelating agent, growth of both strains V7 and V104 was never influenced, strongly suggesting that the concentration of iron is not the direct reason to induce virulency during infection.

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초 록: 비브리오 균에서 확인된 병인물질로서의 외막단백질의 특성

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어류에 비브리오 병을 유발하는 *Vibrio anguillarum*의 병인물질에 대해서는 많은 연구에도 불구하고 아직까지 확실하게 병인물질이 밝혀지지 못한 채, 철분의 흡착물질, 외막단백질, 용혈독소, 그리고 단백질 분해효소 등이 병인 물질이라는 보고가 있었다. 저자들은 병독성에서 차이를 보이는 V7(혈청형 J01) 및 V104(혈청형 J03) 두 가지 균주를 비교 연구하여, *V. anguillarum*이 숙주의 혈청에 대한 저항성, 단백질 분해활성, 세포장해효과 등을 통하여 병독성을 나타내고 있다고 추정하게 되었고, 이들을 유발하는 물질을 규명하는 과정에서 배양 온도에 따라 병독성의 차이가 있으며 온도 변화에 따른 병독성과 외막단백질 발현의 차이에서 상관관계가 있다는 것을 확인하여, 세균의 외막에 존재하는 41 kd의 단백질을 병인 물질로서 추적할 수 있었다. 밝혀진 외막단백질은 V7주가 고온(섭씨 32도)에서 배양되었을 때, 병독성이 감퇴하였으며, 섭씨 23도에서 배양하여 유독한 경우에는 높은 혈청저항성, 혈구 응집성, 그리고 단백질 분해활성을 보였다. V104주도 크기가 약간 작은 외막단백질이 발견되었으나, 상대적으로 병독성의 변화와 깊은 연관을 맺을 수 없어 V7주와는 다른 기작을 통하여 병을 유발할 것으로 추측하였다.