General Characterizations of Two Aeromonas sobria Strains Isolated from Sillago parvisquamis

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General Characterizations of Two *Aeromonas sobria* Strains Isolated from *Sillago parvisquamis*

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Abstract

Two strains of *Aeromonas sobria* NS-2 and NS-10 were isolated from a marine fish *Sillago parvisquamis*. Both strains have β-hemolytic activity on human blood agar plates. Several biochemical characterizations were done on both strains. Also PCR (polymerase chain reaction) was conducted for the amplification of the *aer* (aerolysin) gene. Results revealed that, doubling time, optimum pH, optimum temperature and optimum salinity were nearly the same for both strains. The effect of 13 kinds of antibiotics and the enzyme activites were also the same for both strains. In case of PCR, two amplified bands were obtained for the *aer* gene.

INTRODUCTION

Members of genus *Aeromonas* are primarily aquatic organisms found in polluted and unpolluted sea water and sewage. Some *Aeromonas* sp. are pathogenic for fishes, frogs, turtles and human. The genus *Aeromonas* belongs to the family Aeromonadaceae (Tsukamoto *et al.*, 1993). The organisms in this family are straight or curved gram negative rods and are usually motile by a singular polar flagella. Some cells may produce lateral flagella under certain growth conditions. They produce a wide zone of β-hemolysis on blood agar plates after 1 day (Khardori and Fainstein, 1988).

Aeromonas are listed in the 9th edition of Bergey's Manual of Systematic Bacteriology (Popoff, 1984), as four species and were separated into two main groups on the basis of motility. The nonmotile aeromonads are clustered into one species (*A. salmonicida*) with four subspecies while the motile aeromonads are divided into three species (*A. hydrophila, A. caviae,* and *A. sobria*). Most human *Aeromonas* infections involve the motile aeromonads (Ishimura *et al.*, 1988; Alvarado and Boehm, 1989; McGarey *et al.*, 1991;

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Esteve *et al.*, 1993). Most isolations were in the summer months. The investigators used a semi-selective blood agar plate with ampicillin (10 mg/l) for isolation, and the identification tests were done at 36℃ with a well-defined control (Dixon *et al.*, 1990; Klein and Boehm, 1994; Chung *et al.*, 1995). One case of *A. sobria* bacteria and one case of a cholera-like illness caused by enterotoxigenic *A. sobria* (Campsaur *et al.*, 1982) have been described. Daily *et al.* (1981) have suggested that *A. sobria* may be more important human pathogen than *A. hydrophila* and that *A. hydrophila* is associated more often with environmental isolates.

A. sobria produces several extracellular enzymes with presumed toxic activity, namely diastase, lipase, DNase, lecithinase, elastase, and hemolysin (*e. g.* β-hemolysin) (Nakano *et al.*, 1990; Nieto and Ellis, 1991; Rodriguez *et al.*, 1993). Beta-hemolysin (aerolysin, cytolitic factor) has a molecular mass of 49–53 kd and is released toward the end of the logarithmic phase of growth. It is heat labile, with its activity stopped by heating for 1 h at 50℃ (pH 7) or at 37℃ (pH 8.2). Beta-hemolysin is not destroyed by the proteolytic enzymes trypsin and pronase. It is lethal to rats, mice, and rabbits and causes dermonecrosis in rabbit skin. It is cytotoxic to several cell systems, including human diploid lung fibroblasts (Shieh, 1988 ; Schubert, 1991). The relationship of *Aeromonas* toxins to cholera toxin and to heat-labile *E. coli* enterotoxins is also controversial. James *et al.* (1982) suggested that there are both heat-stable and heat-labile *Aeromonas* toxins, the latter being cross-reactive with cholera antitoxin. Due to the high pathogenesis of *A. sobria* (Merino *et al.*, 1993), in the present study our principal objective is: To conduct some genetical and biochemical studies on two *A. sobria* strains that show β-hemolytic activity on human blood agar plates. These strains were previously isolated from the stomach of marine fish *Sillago parvisquamis*.

MATERIALS AND METHODS

Bacterial strains

Two *Aeromonas sobria* strains; *Aeromonas sobria* NS–2 and *Aeromonas sobria* NS–10 were used in this study. These two bacterial strains were isolated from the stomach of the marine fish *Sillago parvisquamis* which is reported by the another paper in this issue. The bacterial strains have a strong hemolytic activity for human blood erythrocytes and were identified by using ID TEST, EB–20 kit (Nissui Pharmaceutical Co. Ltd., Tokyo).

Measurement of doubling Time

To calculate the doubling time of the bacteria, 10 ml of peptone broth medium was prepared in 50 ml Erlenmeyer flask for each bacterial strain.

This broth medium contains the following (g/l) : polypeptone, 5; yeast extract, 2; NaCl, 25; $MgSO_4$, 1; pH 7.2. The peptone broth medium was inoculated with two loopful of

the bacterial culture slant and shaken in water bath incubator overnight at 25 ℃ with reciprocal shaking at 120 r.p.m. Subsequently, 1 ml of the overnight culture was transferred into a sterile empty tube and was diluted with sterile peptone broth medium until its optical density (O. D.) was adjusted to 0.1 at 660 nm. Then 0.1 ml of that adjusted O.D. culture was transferred to 9.9 ml of sterile peptone broth medium in a L-shaped tube. The O. D. value of each strain was measured every hour for 24 h to plot the growth curve pattern of that bacteria. Also samples of each bacterial culture were counted by using a compound microscope to determine the cell number which corresponds to a particular every optical density. Finally the doubling time of each bacterial strain was calculated.

Effect of pH

To study the effect of pH on the growth rate of the bacterial strains, 0.1 ml of bacterial culture whose O. D. was previously adjusted to 0.1 at 660 nm was added to 9.9 ml of peptone broth medium in L-shaped tube. The pH of these tubes was adjusted by adding 1M of HEPES buffer whose pH value was adjusted to the desired pH gradient. Seven pH gradients 4.4, 5.5, 6.0, 7.2, 8.0, 9.0 and 10.0 were chosen. The O. D. measurements were taken for every pH gradient after 4, 6, 8, 10, and 24 h. Finally, the effect of pH gradient was plotted against the O. D. value to detect its effect on the growth rate. This experiment was conducted in water bath incubator at 25℃ with a reciprocal shaking of 120 r.p.m.

Effect of temperature

The effect of temperature on the growth rate of *Aeromonas sobria* was examined by using peptone broth medium inoculated with 0.1 ml of bacterial culture whose O. D. was adjusted. The pH of the medium was 7.2 and the culture was incubated at defferent temperature levels; 15, 25, 28 and 37 ℃. The O. D. of these cultures was measured after different incubation periods; 4, 6, 8, 10, and 12 h.

Effect of salinity

Different NaCl concentrations were used in this experiment; 0.1, 0.5, 1.0, 2.5, 4.0, 5.0, 7.0, and 9.0%. The same procedures were performed as in the case of temperature and pH. The incubation temperature was set at 25℃, pH 7.2 and the O. D. was measured after 4, 7, 14, 18 and 24 h.

Antibiotics and their inhibitory effects

To test the antibiotic effect on the growth of *Aeromonas sobria*, peptone agar plates were prepared (1.5% agar). Subsequently soft peptone agar (0.5%) was prepared and cooled to 50−55℃. Five drops of overnight bacterial cultures were added to the tubes containing the soft agar. After mixing, the soft peptone agar was poured quickly over the

agar plate surface and shaken gently until forming a bacterial layer over the peptone agar surface. After solidification, 13 different antibiotic discs were placed carefully over the bacterial layer (every plate four discs). Finally the plates were incubated at 25℃ overnight to measure the diameter of the inhibition zone around each disc. The sensitivities of that bacteria to a particular antibiotics is confirmed as the diameter of the inhibition zone.

Apizyme test

To estimate the enzymatic activity of the *A. sobria* strains, an overnight culture for the two strains of *Aeromonas sobria* was performed. Three hundreds ml Erlenmeyer flasks were prepared containing 20 ml of sterile distilled water, 0.9% NaCl and 2 % NaCl solution. Each flask of those three was inoculated by 0.2 ml of the overnight culture. Subsequently, an Apizyme kit was used. This kit (Api Bio Mérieux sa au Capital de 45068400 F/ imprimé en France) contains nineteen enzyme reactions, as follows: phosphatase alkaline, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, phosphatase acid, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and fucosidase and their corresponding substrates are as follows: 2-naphthylphosphate, 2-naphthylbutyrate, 2-naphthylcaprylate, 2-naphtylmyristate, L-leucyl-2-naphthylamide, L-valyl-2-naphthyl-amide, L-cystyl-2-naphthylamide, N-benzoyl-DL-arginie-2-naphthylamide, N-glutaryl-phenylalanine-2-naphthylamide, 2-naphthylphosphate, Naphthol-AS-BI-phosphodiamide, 6-Br-2-naphthyl-α-D-galactopyranoside, 2-naphthyl-β-D-galactopyranoside, Naphtol-AS-BI-βD-glucronic acid, 2-naphthyl-α-D-glucopyrano-side, 6-Br-2-naphthyl-β-D-glucopyranoside, 1-naphthyl-N-acetyl-β-D-glucosaminide, 6-Br-2-naphthyl-α-D-mannopyranoside and 2-naphthyl-α-L-fucopyranoside, respectively. Every kit sheet contains 19 slots. Every slot contains the corresponding enzyme-substrate, and one more slot as a control. Three sheets were used for every bacterial strain, one for bacteria suspended in sterile distilled water, the second for bacteria suspended in 0.9% NaCl and the third for bacteria suspended in 2 % NaCl. Three drops from every flask were pipetted in each slot of the flask-corresponding sheet. All sheets were incubated at 25℃ in a dark room for at least 4h. After incubation, one drop from reagent A; (Tris-hydroxymethyl-aminomethane 250g, sodium dodecyl sulfate 100g, distilled water 1l, pH 7.6–7.8.) was added to all the slots, and one drop of reagent B; (Fast blue BB 3.5g/l dissolved in 2-methoxyethanol) was also added.

All sheets were kept for five min at room temperature, and then transferred to direct illumination for 20 sec. After subjection to light, the color of the reactions developed in the sheets with different degrees of darkness. By comparing the colors of that reaction with a kit chart, numbers and concentrations of enzymes that exist in those bacteria can be recognized.

Isotation of DNA

The method of Saito and Miura (1967) was employed for DNA isolation from *Aeromonas* strains. One hundred ml of peptone broth was inoculated with bacteria and shaken overnight at 25℃ pH 7.2 and reciprocal shaking about 120 r.p.m. All the bacterial cultures were centrifuged at 5000 r.p.m. for 5 min at 4℃ to collect the bacterial cells. One gram of these bacterial cells was used for DNA isolation. Subsequently, 0.8 ml of 0.15 M NaCl-0.1 M EDTA (pH 8.0) was added to the bacterial cells. Also, 10mg/ml of lysozyme dissolved in 0.2 ml of 0.15 M NaCl-0.1 M EDTA was added to the bacterial suspension for the lysis of bacterial cells. The mixture was incubated at 37℃ for 10-20 min. After incubation, 10 ml of 0.1 M Tris-HCl (pH 9.0) -1 % SDS - 0.1 M NaCl was added to the reaction mixture. Also, 10 ml of phenol saturated with 0.1 M Tris-HCl (pH 9.0) was added to the mixture and carefully mixed. After mixing, all the mixture was centrifuged at 5,000 r.p.m. for 5 min. The mixture separated into two layers, the upper layer was transferred to another tube and chloroform was added to remove the phenol. The volume of the chloroform was the same volume as the upper layer volume. After mixing chloroform the tubes were centrifuged again and the upper layer was transferred to another tubes and 2 volumes of cold ethanol was added. The mixture was incubated at − 80℃ for 15 min and was centrifuged at 12,000 r.p.m. for 5 min. The precipitate was collected and washed with 70% ethanol. Finally, the ethanol portion was removed and the precipitate was dried and dissolved in 100 ml of 10-1 TE buffer (Tris- EDTA) for further DNA studies.

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a very important technique. It enables producing enormous numbers of copies of a specified DNA sequence. Also, PCR product can be sequenced directly *i. e.* the specific part of the DNA responsible for producing hemolysin protein can be amplified. In this technique, the template DNA was purified from the RNA that might be present in the DNA sample. The dried isolated DNA was dissolved in 100 μl of 10-1 TE buffer, and 1 μl of RNase was added. The sample was incubated at 37℃ for 30 min. This temperature is optimal for enzyme activation. After incubation, 200 μl of cold ethanol was added and the sample was incubated at − 80℃ for 15 min. Subsequently, the sample was centrifuged at 12,000 r.p.m. for 5 min and the precipitate was dried by aspirator and dissolved in 10 μl of 10-1 TE buffer for PCR reaction. This 10 μl of the isolated DNA was mixed with 10 μl of 10 × reaction buffer, 8 μl of dNTP (deoxynucleotide triphosphates) mixture, 1 μl of primer 1 that has the following sequence 5'> GTC TGT GCC AGT GGT TAT CG<3', 1 μl of primer 2 that has the following sequence 5'>AGA TCC ACA CTG GTA AGC GC<3' (The primers used for this reaction were purchased from Sawady Technology Co. Ltd., Japan), 0.5 μl of *Taq* enzyme and 69.5 μl of sterile distilled water, so the total reaction volume became 100 μl. The PCR mixture was prepared in 0.2 ml tubes, these tubes were put into the PCR machine to perform the reaction. This reaction depends on the preheating of the mixture to 94 ℃ for 1 min, and after that, cycle

reaction will take place. The temperatures which were included in the cycle were as follows : 94 ℃ for 1 min to create a single stranded DNA, 60 ℃ for 1 min to anneal the primer with the template DNA, and the next temperature is 72 ℃ for 1 min, and this temperature is optimal for heat-stable *Taq* DNA polymerase enzyme. This cycle was repeated 35 times and finally the reaction mixture was kept at 4 ℃ until agarose gel electrophoresis will be performed.

RESULTS

Doubling time of *A. sobria* strains

It was found that the doubling time of *A. sobria* strain NS-2 is 19.06 min and that of strain NS-10 is 17.4 min (Table 1). That indicate high rate of growth of strain NS - 10. Also, these two strains reached their mid-log phase after about 5 h starting from 0 time inoculation, and reached their pre-stationary phase after 10 h from 0 time inoculated culture. It can be concluded that as well as these two bacterial strains are very easy in their analysis due to their short life span. They are also very dangerous if they infect any host due to their rapid growth rate.

Effect of pH

Every bacterial strain has its own optimal pH. Some strains have a range for their optimal pH and others have tolerating ability against acidic or alkaline pH. In the present study, the O. D. value with different incubation times (*i. e.* growth rate) of the two strains under different pH values were measured. It was found that, in the case of strain NS - 2 no growth was detected at pH 4.4, while at pH 5.5, 6.0, 7.2, 9.0 and 10.0 maximum growth rate was detected after 10 until 24 h. However at pH 8, maximum growth rate was attained after 10 h and later growth was inhibited. In the case of strain NS - 10, at pH 4.4 low growth rate was detected after 24h incubation. At pH 5.5 and 6.0 the maximum growth rate was found to be after 24 h, while at pH 7.2, 8.0, 9.0, and 10.0 the maximum growth rate was found to be after 10 until 24 h. In general it can be concluded that the growth rate of both strains increased with pH within the range from 7.2 to 9.0 (Table 1).

Table 1 General characteristics of *A. sobria* NS-2 and *A. sobria* NS-10

Characters	NS-2	NS-10
Doubling time	19.06 min.	17.4 min.
Optimum temperature	25℃	25℃
Optimum pH	7.2～9	7.2～9
Optimum salinity	2.5%	2.5%

Effect of temperature

The exposure of the bacterial culture to different temperatures is very important to determine the optimal temperature which is the optimal one for the growth of a particular bacteria. In this study, the growth rate was measured at four chosen temperatures (15, 25, 28 and 37 ℃). It was found that both strains have the same growth pattern. At 15℃ no growth had been detected for both strains until 12 h. After 4 h of incubation the highest growth rate was found to be at 37℃, and after 8h at 28℃ while after 12 h at 25℃. Since at 12 h of incubation is the late log phase for both strains, it was concluded that 25 ℃ is the optimum temperature (Table 1).

Effect of salinity

The concentration of NaCl in culture medium of marine bacteria is an important growth factor because marine bacteria can not live in NaCl free medium. In this experiment the effect of different NaCl concentrations on the growth rate of both strains were studied. The growth pattern for both strains was nearly the same different NaCl concentration. The maximum growth rate for both strains was found to be from 10 to 12 h at all NaCl concentrations and the optimum growth rate was detected at 2.5% NaCl (Table 1). In general there is a linear relationship between bacterial growth rate and salinity.

Effect of antibiotics

The effect of thirteen antibiotics were tested against the two strains. It was found that both strains were completely resistant to ampicillin, bacitracin, lincomycin, and methicillin. Likewise both were completely sensitive to chloramphenicol and novobiocine but sensitive with turbid zone against phosphomycin, erythromycin, colistin, and tetracycline. Also they are sensitive with both clear and turbid inhibition zones against polymixin and kanamycin. However they were weakly sensitive against cefotaxime. This test clarifies that there was no great differences between these two strains in their resistance or sensitivity against the same antibiotics were observed. There were various type of antibiotics that inhibited the growth of both strains (Table 2).

Apizyme test at different NaCl concentrations

This test was conducted to determined the number and concentration of the enzymes produced by the two isolated strains in distilled water (D. W.), 0.9 % NaCl and 2 % NaCl. It was found that only five enzymes were present in these two strains but are different concentrations. Phosphatase alkaline and phosphatase acid activity in case of strain NS-2 were higher than that of strain NS-10 at different NaCl concentrations. Esterase lipase activity in both strains at 0.9% and 2% NaCl was nearly the same. However in distilled water, it was more than 5 nM in the case of strain NS-10 while it was no present in strain

Table 2 Antibiotic effect on strains of NS-2 and NS-10

Antibiotics	Diameter average of the inhibition zone (cm)		Observations
	NS-10	NS-2	
Ampicillin	R	R	R
Chloramphenicol	2.0	2.2	All C
Phosphomycin	1.2	1.2	All T
Erythromycin	1.2	1.3	All T
Colistin	1.3	1.3	All T
Tetracyclin	0.9	1.0	Very T
Bacitracin	R	R	R
Polymxin	1.5	1.5	Only 0.2 cm C
Cefotaxime	1	1.5	Very weak T
Lincomycin	R	R	R
Kanamycin	1.5	1.4	0.5 cm T & 1 cm C
Methicillin	R	R	R
Nivobiocine	1.1	1.1	All C

R, competely resistant. C, clear zone. T, turbid zone.

NS-2. Leucine arylamidase and phosphoamidase activity in distilled water, 0.9 % and 2 % NaCl were the same for both strains. Finally it was concluded that the most abundant enzyme in strain NS-2 and NS-10 was phosphatase acid and has a very high activity at 2 % NaCl, in case of strain NS-2 than in other NaCl concentrations of strain NS-10 (Table 3).

Polymerase chain reaction (PCR)

The aim of performing this reaction is to amplify the part of the DNA responsible for the production of hemolysin which has a degradation effect on the human blood. In this experiment two primers were used. By using the primers, the expected size of the amplified bands of the bacterial DNA is about 1.2 kb. The electrophoresis results revealed that there was two amplified bands, one was 1.2 kb and another was 1.5 kb. Consequently it is expected that there were two amplified sequences of the gene coding for hemolysin activity on the chromosomal bacterial DNA with different sizes. The present result was similar in both strains.

Table 3 Apizyme test for strains of NS - 2 and NS - 10 in different concentrations of NaCl

Enzymes	NS - 2			NS - 10		
	D. W.	0.9% NaCl	2.0% NaCl	D. W.	0.9% NaCl	2.0% NaCl
Phosphatase alkaline	<5	10	20	0	<5	>5
Estrase lipase	0	<5	<5	>5	5	5
Leucine arylamidase	5	5	5	5	5	5
Phosphatase acid	10	20	>40	15	15	15
Phosphoamidase	20	0	10	20	0	10

DISCUSSION

In our study, two *Aeromonas sobria* strains were isolated from the stomach of *Sillago parvisquamis*, and they showed high hemolytic activity. Comparative studies were conducted between them, but the results revealed that there was no big differences between the two strains.

Many investigators worked on *A. sobria* isolated from different sources. Wong *et al.* (1992) isolated *A. sobria* from seafood and aqua culture food available in Taiwan. Incidence of *Vibrio* and *Aeromonas* species in these foods was high. Most of those isolates showed strong lipase or protease activities. Also they were tested for their hemolytic activities, and majority of them were mostly strong.

In our study, *A. sobria* strains isolated from fresh fish showed an phosphoesterase activity (Table 3). They were also tested for their hemolytic activities, and both of them showed a strong β-hemolytic activity against the human red blood cells RBCs, *i. e.* a clear and completely lyzed zone appeared around the bacterial colonies on a human blood agar plates. Lallier *et al.* (1988) described biochemical, toxigenic and surface characteristics of 28 strains of motile *Aeromonas*, 24 strains were isolated from healthy fish, and 4 strains from moribund fish. It was found that the majority of *A. sobria* isolated produced exotoxin, but less than that produced by other *Aeromonas* sp. which were also isolated.

β-haemolysis (β-toxin) is a cell-bound haemolysin, and since our *A. sobria* isolates have this beta hemolytic activity, so it might be probably contain a cell bound β-toxin.

To assess the role of aquaria in the epidemiology of *Aeromonas* associated gastroenteritis, the prevalence and antibiotic susceptibility of aeromonads in ornamental aquaria were determined and compared to that of isolates obtained from patients with gastroenteritis (Joaquin *et al.*, 1989). It was found that thirty eight (76%) of 50 aquaria yielded 61 isolates, 24 (39.3%) of them was *A. sobria.* The aquarium isolates, in contrast to enteric isolates, were generally resistant to chloramphenicol, tetracycline and trimethoprim/sulphamethoxazole. These findings indicate aquaria are unlikely source of aeromonas-

associated gastroenteritis, but their role as possible reservoirs for non-enteric infections with aeromonads needs to be defined.

In case of our isolates the reverse of the previous results were obtained. Both *A. sobria* strains were sensitive to chloramphenicol, ampicillin, bacitracin, lincomycin and methicillin. These results are benefit in controlling the occurrence of *A. sobria* in aquaria, by adding a specific concentration of those antibiotics to inhibit the growth of that bacteria without affecting fishes.

Majeed *et al.* (1990) indicated that certain strains of *Aeromonas* species, in particular *A. hydrophila* and *A. sobria*, are of potential public health significance in meats stored at refrigeration temperature (at 5 ℃ for 7-10 days). In the case of the sampling condition of our study, the fished were freshly bought, and were stored in ice boxes (at 2-3 ℃ for 4-5 h). Our results in the case of *Sillago parvisquamis* stomach indicate that certain strains of Vibrionaceae and Aeromonadaceae have a potentially virulent effect in fished stored at 2-3 ℃ for few hours.

A study was done to detect the gene for aerolysin and the identical genes in *A. sobria* and *A. caviae* by using synthetic oligonucleotide primers in a PCR technique (Pollard *et al.*, 1990). Primers targeted a 209 bp fragment of *aer* (aerolysin) gene coding for the β-hemolysin. The results were negative (no PCR amplification band was detected) in the case of *A. sobria* and *A. caviae* but was positive in the case of *A. hydrophila*.

In the case of our results, we succeeded to amplify the *aer* gene in both strains of *A. sobria* while the expected fragment size is 251 bp. We got two amplified bands, one is 1.2 kb. and the other is 1.5 kb in both strains.

By these results we can conclude that further study can be done on these strain *i.e.* Southern hybridization to blot the *aer* gene and to know if that gene lies on the chromosomal DNA or on plasmid DNA. Likewise, DNA sequencing that gene can be studied and compared with the other sequences of the same gene for other species of Aeromonadaceae.

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