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Characterization of *Aeromonas hydrophila* Isolated from Rainbow Trouts in Korea

Soondeuk Lee, Sookyung Kim, Yoojung Oh, and Yeonhee Lee*

Department of Biology, Seoul Women's University, Seoul 139-774, Korea

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Eight strains of *Aeromonas hydrophila* isolated from diseased trout in Korea were characterized and compared with an American type strain by various methods including biochemical and physiological tests, PCR, randomly amplified polymorphic DNA (RAPD), plasmid profiling, and gel electrophoresis of total, membrane, and extracellular proteins. Virulence factors such as surface array proteins, cytotoxin, hemolysin, haemagglutinin, and protease were also investigated. The Korean strains showed heterogeneity in lysine decarboxylase production, utilization of various carbon sources, and production of acetoin. Five strains had the same profiles of total and membrane proteins. Six strains haemagglutinated with trout red blood cells (RBCs) which was inhibited by fucose, galactose, and mannose, except for No. 1 where haemagglutination was inhibited by only galactose and mannose, but not by fucose. Four isolates haemagglutinated with human RBCs which was inhibited by fucose and mannose yet not by galactose. The type strain haemagglutinated only with trout RBCs which was inhibited by fucose, galactose, and mannose. Every isolate secreted protease, hemolysin, cytotoxin, and siderophore, but no enterotoxin. Results showed that the Korean isolates, except for No. 7, had very different biochemical and molecular characteristics from those of the American type strain.

Key words: *Aeromonas hydrophila*, rainbow trout, virulence factors

It is well established in the fish industry that bacterial infections are responsible for heavy losses in fish farms. Among the etiological agents of bacterial fish disease, the motile *Aeromonas* group, especially *Aeromonas hydrophila*, is considered an important pathogen causing primary infection in wounds or the secondary problem following stress from temperature change, handling, or poor water quality (12, 22). *A. hydrophila* is a rod-shaped, gram negative rod, and facultative anaerobic bacterium (21). This bacterium is responsible for hemorrphagic septicemia, a disease affecting a wide variety of freshwater and marine fish (20) as well as causing a food-borne disease in humans (19). The contamination of trout with *A. hydrophila* can cause a food hazard particularly where there is a possibility of cross contamination with ready-to eat food products. In spite of its wide distribution and pathogenicity, *A. hydrophila* in Korea has not been investigated except for a few studies on strains obtained from a terrestrial source. Since the wide distribution of *A. hydrophila* is probably a consequence of its high capacity to adapt to different environments, it would seem that the genetic and phenotypic diversity of *A. hydrophila* is a natural result (18). To develop a vaccine for trout as well as a detection kit for food-borne diseases caused by *A. hydrophila* in Korea, the isolation and characterization of *A. hydrophila* in Korea is required as the initial step.

In this work, several strains of *A. hydrophila* were isolated from diseased trout, characterized, and then compared with the type strain using various methods including biochemical/physiological tests, PCR, randomly amplified polymorphic DNA (RAPD), plasmid profiling, and gel electrophoresis of total, membrane, and extracellular proteins. Virulence factors such as surface array proteins, cytotoxin, hemolysin, haemagglutinin, and protease were also investigated.

Materials and Methods

Reagents

Media was purchased from Difco (Detroit, MI, U.S.A.). *Taq* DNA polymerase and 500 bp DNA marker were purchased from Takara (Takara Shuzo Co., Ltd., Shiga, Japan). Other chemicals, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

Isolation and identification of A. hydrophila from rainbow trout

Diseased trout were wrapped in plastic wrap and trans-

* To whom correspondence should be addressed.
(Tel) 82-2-970-5664; (Fax) 82-2-970-5669
(E-mail) yhlee@swu.ac.kr

ported in an ice chamber from fish farms (Okdong fishery and Soyang fishery, Kangwon Province) to the laboratory. After the surfaces of the trout were washed with 70% ethanol, the livers and kidneys were obtained asceptically and washed three times with sterile saline. The organs were then homogenized with a tissue tearor (Wheaton, Millville, NJ, U.S.A.) and the homogenate was inoculated onto a BHIA (brain heart infusion agar) and incubated at 28°C. After 2 days, every colony was reisolated and subcultured on a new BHIA. The motile, novobiocin-resistant, oxidase positive, and glucose-fermenting colonies were all considered as *Aeromonas* spp. and further identified.

Characterization by biochemical and physiological tests

The isolates were further characterized by an API 20NE system (Analytab Products, Plainview, NY, U.S.A.) following the procedure as described in the instruction manual.

Identification of A. hydrophila by PCR

PCR was performed with the primers targeted to 16S rRNA and lipase genes in *A. hydrophila* as designed by Dorsch *et al.* (9) and Cascon *et al.* (7), respectively. Each reaction included a negative control to rule out contamination. The PCR products were analyzed on a 1% agarose gel and visualized with ethidium bromide.

Total protein profiling

Bacterial cells which were grown in BHI for 18 h at 28°C with shaking were harvested by centrifugation (8,000 × *g*, 10 min) and washed with 25 mM Tris-HCl (pH 8.0). The proteins were extracted from the cells by boiling in a boiling water bath and then dispersed in 1% SDS/25 mM Tris-HCl (pH 8.0) for 10 min, separated on a 10% SDS denaturing polyacrylamide gel, and visualized with 0.1% Coomassie brilliant blue R-250.

Membrane protein profiling

The bacterial cells at the log phase were harvested by centrifugation, dispersed in 25 mM Tris/Cl (pH 8.0), and broken in a French pressure cell (SLM-AMINCO, Rochester, NY, U.S.A.). The unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was then centrifuged at 100,000 × *g* for 1 h and the pellet containing the membrane parts was suspended in 25 mM Tris-HCl (pH 8.0). The proteins were extracted from the membranes with 1% SDS by boiling in a boiling water bath, electrophoresed, and then visualized as described above.

Extracellular protein profiling

The bacterial cells were inoculated by spreading on to a sterile dialysis membrane laid on a BHIA. After 18 h at 28°C, cells which had grown on the surface of the dialysis membrane were suspended in 3 ml of 20 mM Tris-HCl (pH 8.0) and collected by centrifugation at 10,000 × *g* for 30 min. The supernatant contained the extracellular proteins.

Protein assay

The protein concentration was determined with a BCA (bicinchoninic acid) assay kit using bovine serum albumin (BSA) as the standard.

Haemagglutination (HA) assay

The method of Singh and Sanyal (25) was used with some modification. Red blood cells (RBCs) of human blood type O stored in Alsever's solution (20.5 g D-glucose, 8.0 g trisodium citrate dihydrate, 4.2 g sodium chloride/L, pH was adjusted to 6.1 with 10% citric acid) were collected by centrifugation (300× *g*, 10 min, 4°C), washed three times with PBS, and then suspended in the same buffer making a 3% RBC suspension. The bacterial cells grown in BHI at 28°C for 18 h were washed twice in PBS and then suspended in PBS to yield 10^9 CFU/ml. Ten μl of the 3% RBC suspension and 10 μl of the bacterial suspension were dropped on a slide glass and mixed gently by rocking. The isolate was considered haemagglutination (HA)-negative if agglutination did not occur within 5 min. The sensitivity of the HA to sugars (D-mannose, L-fucose, or D-galactose) was determined by adding 10 μl of 1% sugar in PBS to HA test mixture.

Siderophore production

Siderophore production was observed on a CAS agar plate as described by Schwyn and Neilands (24).

Assay of cytotoxicity

Cytotoxicity was assayed following the procedure used by others (14). If Vero cells had produced, destroyed, or vacuolated the monolayers, the isolate was considered cytotoxicity-positive. The cytotoxic titer was expressed as the highest dilution which showed a positive response. Enterotoxicity was examined as described previously (8).

Assay of hemolytic activity

The hemolytic activity with sheep RBCs was observed on an agar plate and that with human and trout RBCs in broth. Each isolate was streaked on a tryptic soy agar (TSA II) containing 5% sheep RBCs. When a clear zone was formed around a colony, it was considered hemolysin-positive. The hemolytic activity with human and trout RBCs was assayed as described by Asao *et al.* (3) with slight modification: human or trout blood was mixed with an equal volume of Alsever's solution and centrifuged at 300 × *g* for 10 min. Precipitated RBCs were washed twice with Alsever's solution and twice with PBS just before use. Fifty μl of the diluted bacterial culture filtrate was mixed with an equal volume of 1% RBC suspension in a microplate and incubated at 28°C for 12 h. After cen-

trifugation at 2,000 × *g* for 5 min, the absorbance of the supernatant was measured at 540 nm. The hemolytic activity was considered to be the highest dilution of the filtrate producing 50% hemolysis. To produce 100% hemolysis, saponin (final conc. 10%) was added to the RBC solution.

Assay of proteolytic activity

The bacterial cells were grown on a sterile dialysis membrane laid on a BHI-skim milk (15%, w/v) agar. Extracellular proteases were obtained as described above and detected following the procedure described previously (5).

Plasmid profile

Plasmid isolation was performed using the alkaline denaturation method (23) and visualized with ethidium bromide.

Randomly amplified polymorphic DNA (RAPD)

The genomic DNA was isolated using a Wizard genomic DNA isolation kit (Promega, U.S.A). RAPD primers were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) as follows: 1, 5' GGTGCGGGAA 3'; 2, 5' GTAGACCCGT 3'; 3, 5' AAGAGCCCGT 3'; 4, 5' AACGCGCAAC 3'; 5, 5' CCCGTCAGCA 3'. The reaction mixture (25 μl) contained the following: 250 μM dNTPs, 50 ng of genomic DNA, 20 pmole of primer, and 1.25 U of *Taq* DNA polymerase in buffer A [10 mM Tris-Cl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$]. Amplification was performed for 40 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 35°C for 2 min, and extension at 72°C for 2 min. The last cycle was extended to 20 min before the reaction products were stored at 4°C. The amplified fragments were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide.

Results and Discussion

Biochemical and physiological test

It is very important to isolate and characterize Korean isolates in order to develop a vaccine for *A. hydrophila* in Korea since *A. hydrophila* have numerous genomic variations. Eight isolates were selected randomly from several hundred colonies isolated for two years that exhibited characteristics of *A. hydrophila* including motile, novobiocin-resistant, oxidase positive, and fermenting glucose. Isolates No. 2 and No. 4 were not definitively grouped as *A. hydrophila* due to negative lysine decarboxylase. However, both had the ability to utilize L-arginine, which is the characteristic of *A. hydrophila* and thus differentiates them from *A. sorbia* and *A. caviae* (21).

Eight Korean isolates and American-type strain showed diversity in the following tests (Table 1): lysine decarboxylase, citrate utilization, acetoin production, and utilization of sorbitol, rhamnose, sucrose, amygdalin, and arabinose. All Korean isolates and the type strain showed different results in citrate and sucrose utilization. The type strain could use citrate but no sucrose whereas all the Korean isolates could not utilize citrate yet could use sucrose. Since *A. hydrophila* exhibits various biochemical characteristics, the isolates had to be identified by PCR as mentioned previouslly (7).

Table 1. Biochemical and physiological characteristics of isolates

Test	Isolate No.								
	Type strain	1	2	3	4	5	6	7	8
β-galactosidase	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	+	+	-	+	-	+	+	+	+
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-
Citrate utilization	+	-	-	-	-	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-
Tryptophan deaminase	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+
Acetoin production	+	+	-	+	+	+	+	+	+
Gelatinase production	+	+	+	+	+	+	+	+	+
Utilization of glucose	+	+	+	+	+	+	+	+	+
mannitol	+	+	+	+	+	+	+	+	+
inositol	-	-	-	-	-	-	-	-	-
sorbitol	-	+	-	-	-	-	-	-	-
rhamnose	-	-	-	-	+	+	-	-	+
sucrose	-	+	+	+	+	+	+	+	+
melibiose	-	-	-	-	-	-	-	-	-
amygdalin	+	-	+	+	-	+	+	+	+
arabinose	+	+	+	+	+	+	-	+	+
oxidase	+	+	+	+	+	+	+	+	+
NO_2 production	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Growth on MacConkey	+	+	+	+	+	+	+	+	+

PCR specific to 16S rRNA and lipase genes in A. hydrophila

PCR with 16S rRNA targeted primers (Fig. 1a) was performed and all the Korean isolates along with the type strain produced a fragment with an anticipated size of 685 bp (9). When PCR was performed with lipase gene specific primers, a 760 bp fragment was produced in six of the isolates and the type strain with the exception of Nos. 2 and 3 (Fig. 1b). The results of the PCR with primers specific to 16S rRNA and the lipase gene did not correspond with each other. It appears that PCR with primers specific to the lipase gene was more specific than PCR with primers specific to 16S rRNA.

RAPD

Randomly amplified polymorphic DNA (RAPD) techniques have been successfully used to demonstrate simple and reproducible DNA fingerprinting (30). The genomic vari-

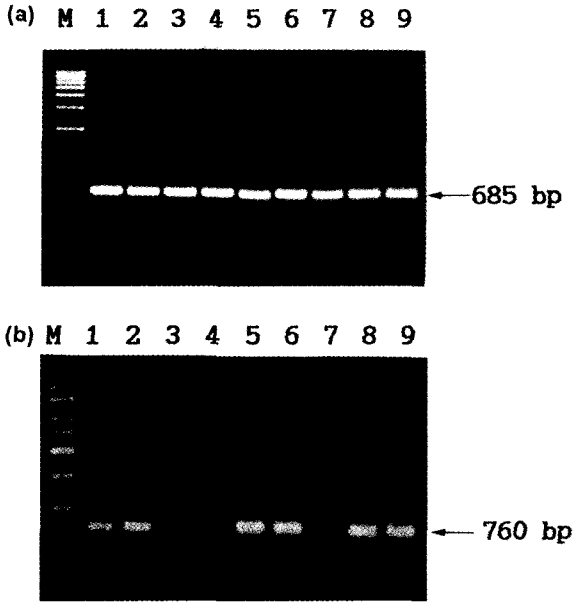

Fig. 1. PCR with primers specific to *A. hydrophila*. PCR was performed with primers specific to 16S rRNA (a) and lipase gene (b). PCR products were electrophoresed using a 0.8% agrose gel and visualized with ethidium bromide.

ation between bacterial species plus the genomic polymorphism between bacterial isolates can be identified by the differences in the sizes and numbers of DNA fragments. RAPD profiles of eight Korean isolates and the type strain produced various DNA fragments, yet no species-specific fragments (Fig. 2). These scattered RAPD profiles highlight the genomic variation of *A. hydrophila* as previously reported (18).

Protein profiling with SDS denaturing gel electrophoresis

To compare their phenotypic characteristics, the profiles of the total proteins, membrane and extracellular proteins were analysed by SDS-PAGE. All strains produced two major proteins with molecular weights between 45 and 52 kD. Isolates No. 1, 4, 6, 8, and 9 showed nearly similar total protein profiles and the remaining isolates revealed no differences. The differences in the total protein profiles among the strains were not clear compared to those of the membrane and extracellar proteins. The type strain and Nos. 3, 7, and 8 produced two major membrane proteins with 46 kD and 38 kD and in the case of No. 5, it produced an additional 15 kD protein. These membrane proteins expressed in every isolate may be involved in the common virulent mechanism such as attachment and could be used as a vaccine. All isolates secreted a protein with 28 kD and in the case of No. 2, produced various extracellular proteins with a wide molecular weight range. The difference in the extracellular proteins suggests a different pathogenicity among the isolates.

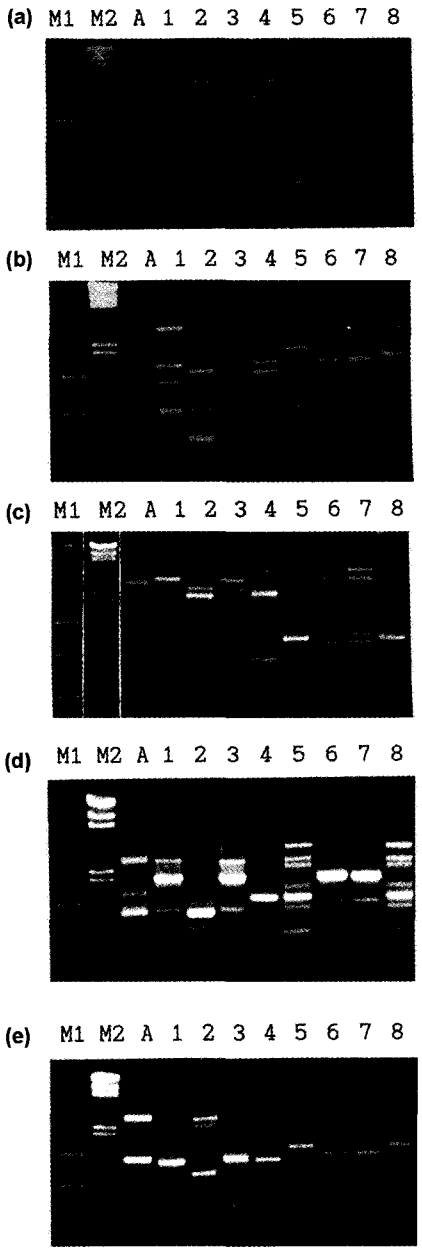

Fig. 2. RAPD of isolates. PCR was performed with various primers and the products were electrophoresed on a 0.8% agarose gel and visualized with ethidium bromide. (a), primer 1; (b), primer 2; (c), primer 3; (d), primer 4; (e), primer 5.

Virulence factors

The ability of *A. hydrophila* to cause a wide range of infections in humans and animals suggests a complex pathogenic mechanism that involves protein toxins (20, 28). Various putative virulence factors of *A. hydrophila* have been demonstrated in an effort to explain the process of pathogenicity. Those factors include hemolysin, aerolysin, cytotoxin, enterotoxin, haemagglutinin, siderophore, surface array proteins, and other enzymes such as protease and elastase (2, 10, 13, 15, 17, 20, 29). Adherence to the

Table 2. Haemagglutination test

Isolate No.	Human blood type O RBC		Trout RBC	
	Agglutination	Inhibited by	Agglutination	Inhibited by
Type strain	-	*[a]	+	F+G+M+
1	+	F+G-M+[b]	+	F-G+M+
2	-	*	-	*
3	-	*	-	*
4	-	*	+	F+G+M+
5	+	F+G-M+	+	F+G+M+
6	+	F+G-M+	+	F+G+M+
7	-	*	+	F+G+M+
8	+	F+G-M+	+	F+G+M+

[a]not applicable because of no agglutination; [b]fucose, galactose, and mannose

host's epithelium has been recognized as an important initial step in bacterial infection. This is often mediated by fimbriae which recognizes specific receptors in epithelial cells. A practical approach to study the various types of fimbriae is to evaluate either the haemagglutination capacity or the competitive inhibition of the reaction using receptor analogues (22). Four isolates (Nos. 1, 5, 6, and 8) were HA-positive with both trout and human RBCs whereas the type strain and two isolates (Nos. 4 and 7) were HA-positive only with trout RBCs. Two isolates (Nos. 2 and 3) were HA-negative with both human and trout RBCs. The HA with human RBCs was inhibited by fucose and mannose, yet not by galactose (F+G-M+), whereas with trout RBCs, the HA was inhibited by all three showing an F+G+M+ pattern except for No. 1 which showed an F-G+M+ pattern (Table 2). This difference in the inhibition pattern may be due to the difference in the receptors in human and trout RBCs. If an organism can recognize specific receptors, it will then have the ability to colonize the host with a subsequent elaboration of tissue damaging toxins that can facilitate penetration into the host and the establishment of infection. Therefore, it is not only the adhesive factor but also the enzymatic capabilities that are essential for pathogenicity (29).

Another virulence factor is the ability to scavenge required nutrients such as iron. Many pathogenic bacteria utilize iron uptake pathways, such as the production of siderophore to access iron for growth. These uptake pathways are considered to be one of the virulence factors, since they facilitate the growth of pathogenic bacteria and the subsequent production of other virulence factors (1, 4, 16, 27). When the isolates were grown on a CAS agar, where siderophore production can be detected, all isolates produced a clear zone.

Other virulence factors such as cytotoxin, enterotoxin, and hemolysin were also examined. Although the titers of cytotoxicity and hemolysin were various among the isolates, there was no significant difference between the titers of the type strain and the Korean isolates. In contrast to

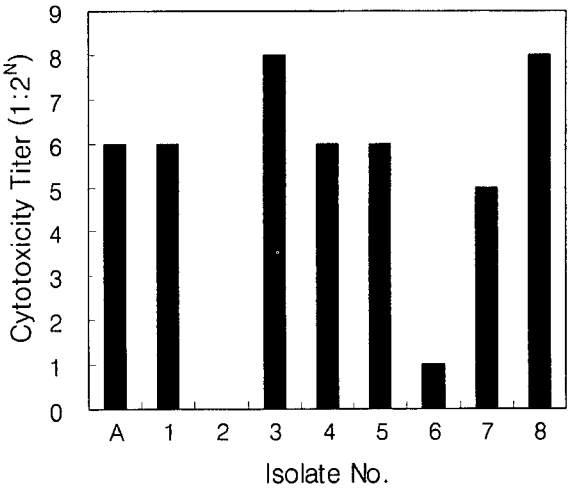

Fig. 3. Cytotoxic activity of the culture filtrate. The culture filtrate of each isolate was serially diluted two fold in PBS and then 50 μl of it was added to Vero cells. After 9 h, the cells were observed under a microscope and the cytotoxic activity was expressed as the highest dilution which showed the cytopathic effect.

the results of Vadivelu *et al.* (28), which suggested a relation between the production of cytotoxin and hemolysin, the results in this study did not show a correlation between these two factors. However, cytotoxicity and hemolytic activity appear to be significantly correlated with acetoin production; the isolates which produced acetoin exhibited both cytotoxicity and β-hemolytic activity. The cytotoxicity level of every isolate, except for Nos. 2 and 6, increased as the incubation time increased (Fig. 3), while the culture filtrate of No. 2 did not show any cytotoxicity and No. 6 showed very little activity. When the supernatant was heated at 56°C for 20 min to destroy

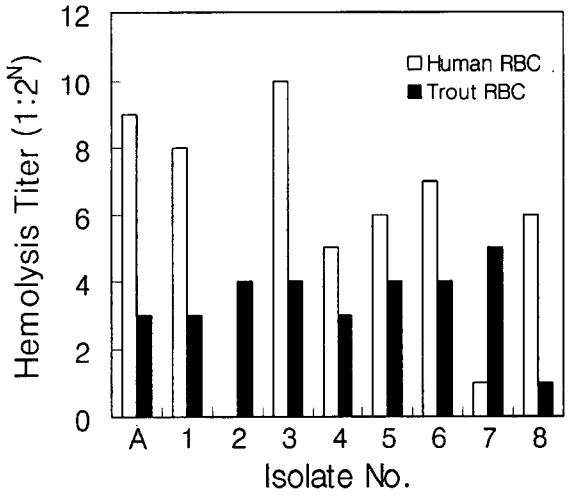

Fig 4. Hemolytic activity of the culture filtrate. Human or trout RBCs were incubated in the presence of a serially two-fold diluted culture filtrate at 30°C for 16 h. The highest dilution which showed 50% hemolysis was determined as the hemolysis titer.

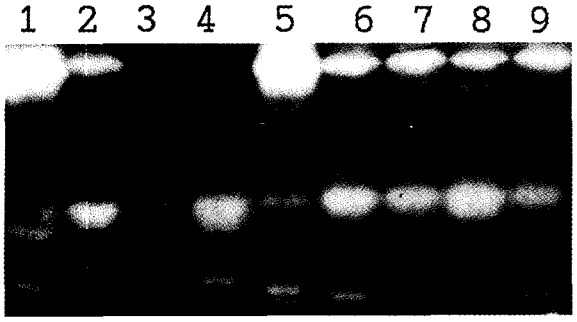

Fig. 5. Proteolytic activity of the culture filtrate detected on a native gel. The culture filtrate was electrophoresed on a 7.5% SDS-polyacrylamide copolymerized with skim milk. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and the bands with proteolytic activity were white due to the degradation of skim milk.

cytotoxic activity and then incubated with Vero cells, there was no cytopathic effect, indicating that enterotoxin was not present. When the isolates were grown in the presence of sheep blood, every isolate showed β-hemolysis and hemolytic activity with human or trout RBCs, except for No. 2 which exhibited no hemolytic activity with human RBCs and showed α-hemolysis with sheep blood. The hemolytic activity of every isolate, except for isolate No. 7, was stronger with human RBCs than trout RBCs (from four fold in the case of Nos. 4 and 5 to sixty-four fold in the case of No. 3). In the case of No. 7, the hemolytic activity with trout RBCs was sixteen fold higher than with human RBCs (Fig. 4). Among the various enzymes involved in penetration, protease production was checked. Each isolate secreted several proteases which were able to degrade not only milk protein (Fig. 5), but also gelatin, lysozyme, and immunoglobulin G (data not shown).

Plasmid analysis

Since *A. hydrophila* can be transferred from animals to humans and several bacterial phenotypic properties such as antimicrobial resistance or virulence factors have been demonstrated to be plasmid encoded, the presence of plasmids may present a potential public health hazard. A previous report (11) has shown that the presence of plasmids in clinically important bacteria increases their virulence. In contrast, the presence of plasmids has a negative effect on bacterial virulence factors in some cases. For example, Smith and Tucker (26) reported that the loss of plasmid increased the pathogenicity of some bacteria. In this case, plasmid is thought to be involved in the regulation of genes that are coded for virulence. Six isolates, except Nos. 1 and 4, had a large plasmid of 20 Kbp, however Nos. 1 and 4 did not have any plasmid (Fig. 6). Isolates No. 5 and 8 had an additional plasmid of 4.8 Kbp. Plasmids of similar size have been observed by Vadielu *et al.* (29) and Borrego *et al.* (6). However, a correlation among plasmids, biochemical characteristics, and virulence factors has not been observed. The role of these plasmids may be identified by observing their various characteristics after curing them.

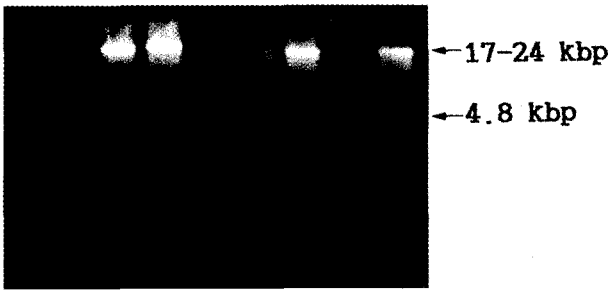

Fig. 6. Plasmid analysis. Plasmids were isolated and electrophoresed on a 0.8% agarose gel and visualized with ethidium bromide.

In sum, the results indicated diversity among the *A. hydrophila* isolates of trout in Korea and great differences from the type strain. Accordingly, these isolates are currently being used to develop a vaccine and detection method for *A. hydrophila* infections in trout as well as food-borne diseases of humans.

Acknowledgments

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