Changes of Outer Membrane and S-layer Protein Profiles of
Aeromonas hydrophila by Starvation

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(Received March 4, 1998)

It was reported in our previous paper that the starved cells of Aeromonas hydrophila were more virulent compared to the cells cultured in a medium. In this study, differences in their protein profiles were compared between the starved and cultured cells of the bacterium. Total proteins, outer membrane proteins (OMP) and S-layer proteins prepared from starved and cultured cells were analysed by SDS-PAGE. A different pattern was shown in the OMPs between the cells. Major bands of OMPs, 39, 52 and 97 kDa, were detected in the starved cells, however they were not detected in the cultured cells. In S-layer fraction, major band estimated at 91 kDa was found only in the cultured cells. Experimental result on phagocytosis of crucian carp macrophages against those cells revealed that phagocytic rate of the starved cells was lower than that of the cultured cells. These results indicate that starvation induces shifts in OMP and S-layer proteins of A. hydrophila which may enhance the resistance of the bacterium against phagocytosis of macrophages.

Key words: Aeromonas hydrophila, starvation, outer membrane protein, S-layer protein, phagocytosis.

Aeromonas hydrophila, a Gram-negative motile rod, is a pathogen of a wide variety of hosts; fish, animals and human. The first step in pathogenesis for a systemic infection with A. hydrophila is colonization on the invading site of the host. The colonization process is associated with the bacterial cell surface, including the production of proteinaceous adhesion (Jones and Isaacson, 1984), certain outer membrane (OM) (Buchanan and Pearce, 1979) and antiphagocytic carbohydrate capsule (Robbins et al., 1980).

A number of bacteria produce paracrystalline arrays of proteins known as S-layers on their surface (Robbins et al., 1980). The S-layers are assembled externally to the outer membrane, by a noncovalent interaction between the OMP and S-layer protein (Messner and Sleytr, 1992; Smith, 1986). It has been demonstrated that the cell surface proteins such as OMP and S-layer protein can be virulence factors of Gram-negative bacteria (Aoki and Holland, 1985; Belland and Trust, 1984; Biosca and Amaro, 1991; Buchanan and Pearce, 1979; Chakrabarti et al., 1996; Dooley et al., 1986; Ishiguro et al., 1981; Kay et al., 1985; Mittal et al., 1980). In the case of A. hydrophila, it has been reported that the OMP and S-layer proteins are related to its virulence (Dooley and Trust, 1988). A. hydrophila is ubiquitously inhabited in the natural aquatic environment (Neilson, 1978). However, there is no information about the cell surface proteins of A. hydrophila in the natural environments. Therefore, it is important to elucidated the profiles of OMP and S-layer proteins when the bacterium was transferred from a cultured condition to natural environments.

We found that a starved A. hydrophila strain was more pathogenic than the cultured one in the previous study (Rahman et al., 1997), where it was hypothesized that in a natural environment or in a starved condition A. hydrophila has different profiles in OMP and S-layer structures and also active participation of these proteins in the resistance against host defense systems such as phagocytic cells. Phagocytosis, the cellular ingestion and digestion of particulate matter, is a widely distributed defense reaction occurring in virtually all animal phyla (MacArthur and Fletcher, 1985). Non-specific, cell mediated immune responses and phagocytic function of fish appear to play a very important role in the protec-
tion against commonly encountered pathogens (Finco-Kent and Thune, 1987). As the phagocytic activity is affected by the surface properties of ingesting particulate matter, it is likely that the OMP and S-layer have effects on phagocytosis.

The aim of the present study was to compare the OMP and S-layer protein of a cultured and starved A. hydrophila and resistance of these two bacterial preparations against fish macrophages.

Materials and Methods

Bacterium

A. hydrophila strain A-3500 originally isolated from a diseased eel Anguilla japonica in Shizuoka Prefecture, Japan, was used for the present study after passed in carp Cyprinus carpio several times to increase pathogenicity. The strain was cultured in nutrient broth (Nissui) at 25°C.

Preparation of cultured and starved cells

The bacterium precultured in nutrient broth at 25°C for 24 h was inoculated in a fresh 1l nutrient broth and incubated at 25°C for 24 h. The cells collected from this culture were used as the “cultured cells”. “Starved cells” were prepared as follows; “cultured cells” were suspended in 0.01 M phosphate buffered saline (PBS) and washed twice by centrifugation at 3,000 ~ g for 20 min. Washed cells (0.5 g, wet weight) were suspended in 10 ml of formulated water (FW) containing 0.6% NaCl, 0.5% KCl, 0.1% CaCl2• 2H2O and 0.2% MgCl2 • 6H2O and incubated for 24 h at 15°C for starvation. FW was selected from the result of the previous study (Rahman et al., 1997). After incubation, cells were harvested by centrifugation at 3,000 × g for 20 min.

Preparation of total protein fraction

The cultured cells and starved cells (0.5 g, wet weight) were washed twice with PBS and once with 10 mm Tris-HCl, pH 7.5. The cells were collected by centrifugation at 3,000 × g for 20 min and suspended in 10 ml of 10 mm Tris-HCl and disrupted by sonication on ice (50 W, 30 s, 4 times). The supernatant obtained after centrifugation at 2,000 × g for 10 min was used as a total protein fraction.

Preparation of OMPs

OMPs were prepared according to the method of Dooley and Trust (1988). The cultured and starved cells (0.5 g, wet weight) were suspended in 10 ml of cold 10 mM Tris-HCl. Then DNase and RNase were added to the suspension and the cells were disrupted by sonication on ice (50 W, 30 s, 4 times). Unbroken cells were discarded by centrifugation at 10,000 × g for 30 min. Total cell envelope fraction was obtained as precipitate of centrifugation at 35,000 × g for 40 min. The envelope pellet was suspended in 1.0 ml of 20 mM Tris-HCl, pH 7.5, containing 10% (w/v) sucrose. The suspension was applied to the top of a tube holded discontinuous density gradient of 20, 30, 40, 50 and 60% (w/v) sucrose in 20 mM Tris-HCl, pH 7.5. Centrifugation at 200,000 × g for 22 h was performed and a 0.5 ml portion was collected from the bottom of the tube. The collected envelope was washed once with 20 mM Tris-HCl, pH 7.5, at 100,000 × g for 40 min and stored at −70°C until used as the OMP fraction.

Preparation of S-layer fraction

S-layer protein was isolated by the method of Dooley and Trust (1988). Cells were suspended in 0.2 M glycine-KOH, pH 4.0, and stirred for 30 min. The cell pellets were discarded by two times centrifugations at 12,000 × g for 20 min and the supernatant was collected. The collected supernatant was centrifuged at 50,000 × g for 45 min and the precipitate was washed once with 20 mM Tris-HCl, pH 7.8, by centrifugation at 50,000 × g and stored at −70°C until use as the S-layer fraction.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970). The concentration of stacking gel and separation gel were 4 and 14%, respectively. Samples were dissolved in 1 M Tris-HCl, pH 6.8, containing 10% (w/v) SDS, 3% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.025% (w/v) bromophenol blue, and the solution was heated at 98°C for 6 min. After electrophoresis the protein bands were visualized by staining with Coomassie brilliant blue (CBB) R-250.

Fish

Crucian carp (Carassius cuvieri), weighing about 25–40 g were purchased from a carp farm in Kochi Prefecture and maintained in 600 l tanks with well aerated water until use. The fish were fed commercial food pellets.

Phagocytosis assay

Ten crucian carp were sacrificed and dissected longitudinally from the ventral side. The head kidney was
removed and washed with 1 ml of Eagle’s minimum essential medium supplemented with 10% fetal calf serum (MEM-10). Then the kidney was cut into small pieces in MEM-10 and kept for few minutes. The obtained cell suspension was centrifuged at 2,000 × g for 2 min and cells were collected. Collected cells were washed 3 times (2,000 × g, 2 min) with the same medium. The leucocytes were counted in a hemocytometer by a light microscope and their viability was determined by trypan blue exclusion. The cell suspension was then adjusted to 5.9 × 10⁶ cells/ml. To prepare macrophages monolayers, 100 µl of this suspension were added to glass coverslips placed in a 24 well culture plate. Two monolayers were prepared from each fish. After allowing 30 min at 25°C for cells to adhere, non-adherent cells were washed away with MEM-10. Then 500 µl of either the starved or cultured bacterial suspensions (5 mg/ml in MEM-10) were added to the coverslips. The 24 well culture plate was then transferred again to a 25°C incubator and phagocytosis was permitted over an hour. Bacterial cells were carefully washed away and the monolayer was fixed with ethanol and stained with Giemsa’s staining solution (Hamaguchi et al., 1989). The number of phagocytosing cells per 200 cells were counted microscopically. The results of phagocytosis were expressed as phagocytic rate (PR), the percentage of cells showing phagocytosis and the phagocytic index (PI), the number of phagocytosed A. hydrophila cells/phagocyte.

**Results**

**SDS-PAGE analysis of total protein, OMPs and S-layer protein**

Remarkably different protein pattern was not observed by SDS-PAGE analysis of total proteins (Fig. 1A) in cultured and starved cells.

However, quite different patterns were shown in the OMP fraction, the starved cells possessing 39, 52 and 97 kDa major bands which were not found in the cultured cells (Fig. 1B). On the other hand, proteins found at around 26, 30, 39, 60 and 70 kDa in the cultured cells were lost in the starved cells. It was found that A. hydrophila synthesized some new proteins and lost some proteins in their outer membrane by starvation.

S-layer proteins stained with CBB showed only one prominent band at 91 kDa in the cultured cells of A. hydrophila which was absent or very faint in the starved cells (Fig. 2).

The phagocytosis of cultured and starved A. hydrophila by crucian carp macrophages over a 60 min period are presented in Table 1. Cultured and starved cells showed a phagocytic rate of 19.52 ± 1.42 and 14.73 ± 1.54 % and phagocytic index 5.14 ± 0.38 and 6.80 ± 0.45, respectively. The difference in the phagocytic rate is statistically significant (P < 0.01). However, there was no significant difference in the phagocytic index.
To compare the characters between the cultured and starved cells of A. hydrophila, profiles of solubilized total proteins and OMPs were analyzed by SDS-PAGE. In the total proteins no obvious difference was observed between the cultured and starved cells, however, separated OMPs showed quite different profiles between them. The result indicates that starved A. hydrophila cells lose some OMPs and synthesize some new proteins in the outer membrane.

Starvation induced a change also in the S-layer fractions. The 91 kDa major band observed in the cultured cells was lost in the starved cells. The S-layer of Aeromonas salmonicida has been reported to contribute to resist against unfavorable conditions (Munn et al., 1982). In case of starvation condition, the S-layer of A. hydrophila may not have an essential role to resist against host defense. The results of the phagocytosis experiment where significant difference was shown in the phagocytic rate may indicate that starved cells have acquired resistance against some kinds of macrophages. Higher, but not significantly, phagocytic index in the starved cell may indicate that starved cells are not easily digested and survive in the macrophages. At any rate it is interesting that after loss of the S-layer protein, starved cells show higher resistance to phagocytosis than the cultured cells. It is hypothesized that due to starvation S-layer protein might be replaced by another surface proteins such as 39, 52 and 97 kDa OMPs which may help the starved cells to resist against host defenses.

Dooley and Trust (1988) reported that S-layer fractions of A. hydrophila was isolated by glycine buffer extraction method and SDS analysis showed that the major protein was 52 kDa. But in our study SDS-PAGE analysis of S-layer materials showed 91 kDa protein in cultured cells of A. hydrophila strain A-3500. At present, it is not clear why different molecular sized proteins were shown in the same species. Studies on structural characteristics of the S-layer proteins are necessary.

Hood et al. (1986) reported that drastic changes took place in the membrane of Vibrio cholerae when starved. Amy and Morita (1983) demonstrated that, starved cells of Vibrio sp. lost many cellular proteins but synthesized new cellular proteins. The newly synthesized proteins were termed as starvation proteins. Nelson et al. (1997) reported that Vibrio anguillarum also synthesized new protein in total proteins due to starvation. These previous reports suggest the importance of study on starved bacteria which may be reflect "natural" bacteria in their cell components.

Rahman et al. (1997) reported that the starved A. hydrophila is more pathogenic than the cultured one. It is also reported that the outer membranes of Gram-negative bacteria may play vital role in virulence of bacteria (Aoki and Holland, 1985; Biosca and Amaro, 1991; Buchanan and Pearce, 1979; Dooley et al., 1986). It is capable to presume that the newly synthesized OMPs of A. hydrophila take part in its virulence. The function of the new proteins which appeared in the starved cells is a clue to clarify the pathogenicity of A. hydrophila.

Acknowledgements
The authors are thankful to Dr. T. Yano, Kyushu University, for providing the bacterial strain and Dr. S. J. Jung, Fish Disease Laboratory, Kochi University, for kind help during this work. The first author is grateful to the Ministry of Education, Science and Culture of Japan for financial support.

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