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Electron microscopic studies on *Flavobacterium branchiophila* in experimentally induced gill disease of rainbow trout

Gang-joon Heo
Department of Veterinary Medicine, College of Agriculture, Chungbuk National University
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細菌性 아가미病에 實驗的으로 感染된 무지개송어에 있어서 *Flavobacterium branchiophila*에 대한 電子顯微鏡學的 硏究

허 강 준
충북대학교 농과대학 수의학과
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초록 : 건강한 무지개송어의 치어와 실험적으로 세균성 아가미병을 감염시킨 무지개송어의 치어에 있어서 아가미 조직과 그 병원균인 *Flavobacterium branchiophila*에 대하여 주사형 전자현미경(SEM)과 투과형 전자현미경(TEM)으로 관찰하였다. *F branchiophila*는 길이 5~8 μm, 폭 약 0.5 μm의, 2~3 균체가 서로 연결된 길고 가는 장간균으로서 균체의 주위에는 다수의 선모(pili)를 갖고 있었다. 세균성 아가미병의 초기병소는 초미세 형태학적으로 이차새변의 비후에 의해 시작되며 병원균은 아가미 표면과 일정한 거리를 유지하며 부착되어 있었다.

주사형 전자현미경의 관찰에서 건강한 물고기의 아가미 표면은 미로와 같은 모양과 미세 섬모상의 돌기로 특정지어 질 수 있는 상피세포가 보이나 실험적으로 감염된 아가미 표면에서는 새변의 선단에서 부터의 증생·융합과 함께 표면의 미세구조가 붕괴되어 잘 관찰되지 않았다. *F branchiophila*는 표면에 실뭉치와 같이 엉클어져 부착되어 있었으며 5% 식염처리후에는 그 형태가 일정치 않았다.

Key words : electron microscope, bacterial gill disease(BGD), rainbow trout, *Flavobacterium branchiophila*.

Introduction

Bacterial gill disease (BGD), which results in high mortality in salmonid fishes, is characterized by the presence of numerous filamentous bacterial cells on the surface of the gills.[1,2] Such gram-negative bacteria have been isolated from some salmonids with BGD in Japan and Oregon, USA and classified as *Flavobacterium* sp.[3,4] This bacterium appeared abundantly on the surface of the gills of juvenile trout within 18 to 24 hours after waterborn exposure of the fish to a bacterial suspension of cells in an aquarium. The infection caused irritation to the gills and impaired their respiratory function.[5] The fish eventually died from suffocation in water containing levels of dissolved oxygen much higher than the incipient lethal levels for uninfected control fish.[5] Farkas[6] isolated filamentous, nongliding bacteria from certain freshwater fishes afflicted with BGD in Hungarian fish farms. The isolates were identified as *Flavobacterium* sp. and were reported to exhibit positive slide agglutination with the antiserum prepared against the isolates from Japan. We presented evidence that BGD isolates from Japan, USA, and Hungary constitute a new species, for which the name *Flavobacterium branchiophila* was proposed.

Strain TS-1(= BGD-7721, ATCC 35035) was designated as the type strain of the new species.[7]

In spite of the controversy concerning the causative agents of the disease, histopathological descriptions have contributed to its understanding.[8,9] The question of proliferation of epithelial cells was of particular interest and attraction. Kudo and Kimura[10] frequently observed fusion at the distal tips of adjoining gill lamellae in diseased trout fingerlings. This was also of great interest in relation to the binding or interaction between epithelial cells. In addition to descriptions of gill filament and lamellar epithelia[11,12] and on secondary lamellae deveolopment,[13] it was of great importance to understand the ultrastructural differences between the cell organization of normal and diseased gill lamellae and filaments. To understand the underdeveloped gill epithelium, especially the gill lamellar epithelium, the use of trout fingerlings as material was signigicant because of the higher susceptibility of these animals to bacterial gill disease.

Before these experiments could be done, however, I found it necessary to have a detailed cytopathological portrait of the steps of the disease and an ultrastructural description of the lesions. The purpose of this paper is to define the ultrastructural differences between gill epithelia before and following bacterial infection, morphological characteristics of the attached causative agent, *Flavobacterium branchiophila*, and the gill epithelium, and morphological change of the bacteria and gill epithelia by treatment using 5% NaCl, and the recovery of hyperplastic lesions in an experimentally induced gill disease.

Materials and Methods

Bacterial strains : *Flavobacterium branchiophila* TS-1(= ATCC 35035) and BGD-7501 were used. These strains isolated from diseased salmonid fish included the following organisms : strain TS-1, which was isolated in 1977 in Gunma, Japan, from yamame(*Onchorhychus masou*);strain BGD-7501, which was isolated in 1977 in Gunma, Japan, from rainbow trout.[4]

Morphology of *Flavobacterium branchiophila* : Preparations of the bacteria were stained with 2% phosphotungstic acid on copper grids coated with collodion and carbon. The excess stain was removed by touching it with a piece of filter paper. The specimens were examined with an electron microscope (JEM-1200EX;JEOL Ltd., Tokyo, Japan) at 80 kV.

Experimental infection : Aquarium of twenty liters in volume containing 5 liters of sterilized water was prepared. After a 2 day acclimatization period, twenty rainbow trout fingerlings, *salmo gairdneri* Richardson, were exposed to bacterial suspension (10^6 CFU/mℓ) for 2 h in static water with aeration, and water temperature was kept at 20℃. After, a successful infection was confirmed by microscopic examination of the gill surface of some fish samples, water in the aquarium was changed.

Immersion of infected fishes in 5% NaCl : The successfully infected fishes were immersed in 5% NaCl for 2 min as a treatment. This procedure was repeated three times at six day intervals.

Transmission electron microscopy : Thirty rainbow trout fingerlings suffering from bacterial gill disease and twenty healthy ones were used for the present investigation. Their average body length and body mass were approximately 39 mm and 1 g, respectively. Gill tissues were excised from fresh fingerlings and fixed for 2 to 3 hrs in 2.5% glutaraldehyde buffered with an ice-cold, 0.1 M cacodylate buffer (pH 7.3) followed by thorough washing with the same buffer containing 5% sucrose and osmication with the buffered 1% osmium tetroxide. The gill tissues were dehydrated by a graded series of cold ethanol and embedded in Epon 812. Ultrathin sections, doubly stained with uranium and lead, I examined by a JEM-1200EX type transmission electron microscope.

Scanning electron microscopy : After fixation for 0.5 to 1 h in cacodylate-buffered 2.5% glutaraldehyde the gill tissues were briefly subjected to supersonic treatment for removal of the mucous substance coating the gill epithelium surface and of the bacterial cells, followed by immersion in the same fresh fixative for 1 to 2 h. After washing overnight with 0.1 M cacodylate buffer (pH 7.3) containing 5% sucrose, the gill tissues were immersed for 2h more in solution made of an equal volume of 2% solution of sucrose, sodium glutamate, glycine and arginine hydrochloride, and then for 2 to 18 h a 2% solution of tannic acid after washing well for 30 min in distilles water. After washing in distilled water, postosmication and gill tissues were transferred to amyl acetate, and then dried at a critical point from liquid CO_2 The dried specimens were mounted on stubs, coated with platinum by ion sputtering and examined by JSM-T100 (JEOL

Ltd., Tokyo, Japan) scanning electron microscope (SEM).

Results

Transmission electron microscopic studies : The cells of *F branchiophila* were gram-negative slender rods measuring 0.5 by 5 to 8 μm, and they usually occurred in chains of two or three cells in Cytophaga broth (Fig 1). The pili were long, thin, flexible filaments measuring approximately 4 nm by 1 μm. The pili were packed together to organize into bundles.

The normal epithelium of gill filaments and lamellae was comprised of a single or double layer of the same types of cells. Cell organization was usually characterized by a mosaic arrangement among the three cell types of flat epithelial, chloride and mucous cells.

In diseased gill epithelium, The bacterial cells attached to the gill surface through pili, but they were slightly separated from the surface or the tips of microvillus-like projections of epithelial, chloride and mucous cells(Fig 2 and 3), and did not invade into gill tissue. The hypertrophy was characterized by morphological changes of epithelial and chloride cells, and the infiltration of wandering leukocytes. The lamellar epithelial cells, especially the outermost ones, were characterized by a columnar or cuboidal shape and thickening and simultaneous obvious decrease of microvillus-like projections.

Scanning electron microscopic study : Normal surface ultrastructure of epithelial cell in the outermost layer were characterized by a roughly squamous or polygonal outline and branching and anastomosing microridges on the cell surface. They ofter exhibited a typical labyrinth-like structure(Fig 4).

Hyperplastic lesions in experimentally infected gill were most serious at near the tips and mild towards the gill arch. Each filament exhibited a club-like or cudgel-like contour, and fusion between the filaments was sometimes observed at their tips. Topographical variations brought out markde differences in the surface appearance of the epithelia which lacked the typical labyrinth-like microridge structure. On the surface of gill filaments, thread-like bactreial cells attached and were entangled (Fig 5). Fig 6 appeared micrograph of the gill epithelium 48 hours after exposure of the fish to suspension of *F branchiophila* TS-1 in an aquarium. The bacterial cells almost covered the surface, have pili around the cell body in an enlarged micrograph (Fig 7). After immersion in 5% NaCl, the cell of *F branchiophila*, however, appeared to be indeterminate shape (Fig 8). Viability of such bacteria was not still uncertain, although plating method did not detected it. Surface ultrasturcture of epithelial cells varied and showed no typical microridge structure except for a few cases of distorted microridges or sparsely granular, roughly broken or irregularly pebbled appearance. The surface ultrastructure was so complex that it was impossible to characterize definitely.

Discussion

Most investigators believed bacterial gill disease to be principally a myxobacterial infection.[14,15] Kimura et al[3] reported the disease was caused by the infection of *Flavobacterium* sp. which had been isolated by them, and they have succeeded not only in reproduction of the disease using the bacterium[9] but also in extraction of a material inducing the disease from the bacterium.[16] However, attempts succeeded in transmitting bacterial gill disease to fingering trout by adding pure cultures of the bacterium in aquarium by Wakabayashi et al.[4] The experimentally infected gills showed essentially the same symptoms as seen in naturally occurred gill disease. Recently, the name *Flavobacterium branchiophila* was proposed for a group of the bacterial strains those which were isolated from cultured salmonids or sheatfish suffering from bacterial gill disease in Japan, USA and Hungary.[7]

As is already well known, the most striking characteristics in bacterial gill disease are hyperplasia of gill epithelia and fusion of adjacent gill lamellae.[9,14,17] Although Wood and Yasutake[9] reported that the bacterial type of hyperplasia frequently developed in the lamellar epithelium and was often detected first at the extreme distal tip of the lamellae, the present observations and other data on experimental infection of bacterial cells[10] have revealed that hyperplasia starts at the distal end of the filaments and progresses towards the proximal portion. This may be closely related to the location of the first adhesion of bacterial cells.

The electron micrograph of the *F branchiophila* revealed that the pili had a tendency to aggregate into parallel bundles. The structures such as bacterial flagella bases

were not observed on the pili filaments. In comparison with the type 1 or common pili of *Escherichia coli* that was characterized by a rigid rod-like shape of 7 nm in diameter,[18,19] the pili of strain TS-1 and BGD-7501 were thinner and flexible. During the course of the histological preparation for the transmitting electron microscopy, many of the bacterial cells fell away from the surface of the gill epithelium. This may have been caused by the discharge of mucous substance from mucous cells.[20] From the fact that *F branchiophila* never invade into gill tissue, it is suggested that the pili plays an important role in the mechanism of the infection.

SEM observations on the surface ultrastructure of gill epithelia in healthy rainbow trout previously revealed a pattern of microridges on the surface of epithelial cells, microvillus-like cytoplasmic projections on the chrolide cell surface and occasionally granular openings on mucous cells.[21,22] However, SEM observations of abnormal epithelia in bacterial gill disease revealed that surface ultrastructure varies most remarkably in epithelial cells. Further, the outermost layer of epithelial cells in lesions showed structural changes of microridges in a uniform direction, varying from the typical, labyrinth-like pattern through distortion or transformation into bead-like chains to decay and disappearance. These findings have been confirmed in a present experimental infection of the disease.

Summary

Gill epithelia of normal rainbow trout fingerlings and abnormal ones suffering bacterial gill disease by experimental infection were examined by transmitting electron microscopy (TEM) and scanning electron microscopy (SEM). TEM observations revealed that *Flavobacterium branchiophila* consisted of slender rods measuring 0.5 by 5 to 8 μm, and they had which were long, thin, flexible filaments measuring approximately 4 nm by 1 μm, and packed together to organize into bundles. Morphological alterations of the diseased epithelia started at hypertrophy of the lamellar epithelium. *F branchiophila* attached to the gill surface of infected fish through pili with a regular distance, and did not invade into gill tissue.

In SEM observations, normal surface ultrastructure of epithelial cell in the outermost layer were characterized by a typical labyrinth-like structure branching and anastomosing microridges on the cell surface. Hyperplastic lesions in experimentally infected gill were most serious at near the tips. Each filament exhibited a club-like, and fusion between the filaments was sometimes observed at their tips. On the surface of gill filaments, thread-like bacterial cells attached and were entangled. The bacterial cells almost covered the surface. After immersion in 5% NaCl, the cell of *F branchiophila*, however, appeared to be indeterminate shape.

Legends for figures

Fig 1. Electron micrograph of negatively stained cells of *Flavobacterium branchiophila* TS-1(=ATCC 35035, BGD 7721). Bar=1 μm.

Fig 2. Contact of *Flavobacterium branchiophila* with a lamellar epithelial cell. Some bacterial cell (B) in a transversal section are in contact with the cytoplasmic projections of the lamellar epithelial cell. Bar=2 μm.

Fig 3. Magnified transmitting electron micrograph of *Flavobacterium branchiophila* on the gill epithelium. Bar=0.5 μm.

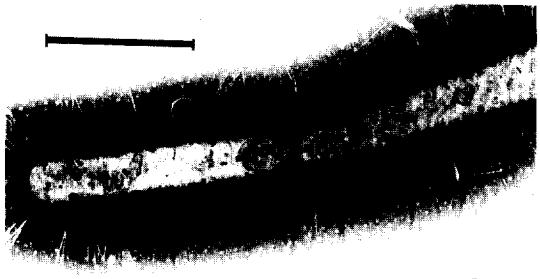
Fig 4. Surface ultrastructure of normal gill filament epithelium. ×2,000.

Fig 5. Scanning electron micrograph of *Flavobacterium branchiophila* on the gill epithelium 24 hours after exposure of the fish to suspension of the bacteria in an aquarium. ×2,000.

Fig 6. Scanning electron micrograph of *Flavobacterium branchiophila* on the gill epithelium 24 hours after exposure of the fish to suspension of the bacteria in an aquarium. ×2,000.

Fig 7. Magnified scanning electron micrograph of *Flavobacterium branchiophila* attached on the surface of gill epithelium, ×35,000.

Fig 8. Electron micrograph of indeterminate shaped *Flavobacterium branchiophila* 24 hours after immersion in 5% NaCl. ×2,000.



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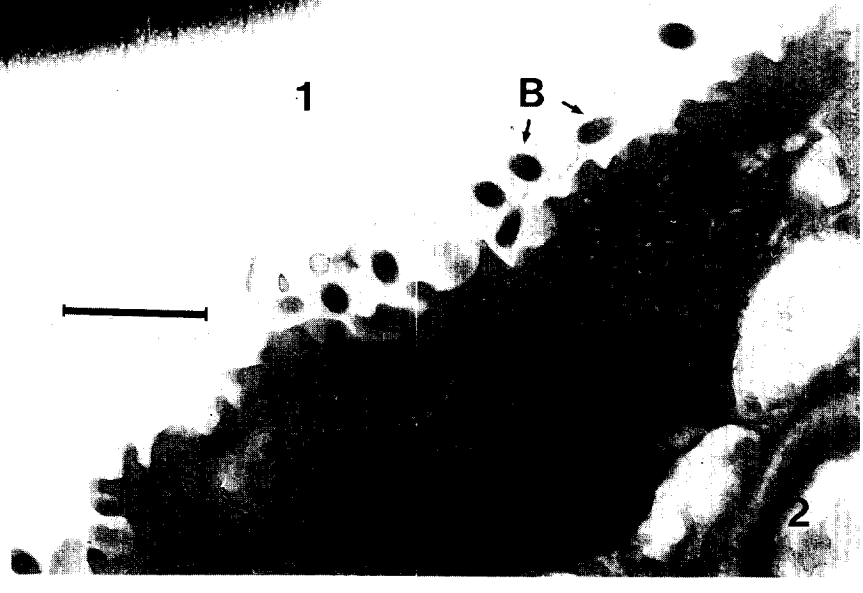

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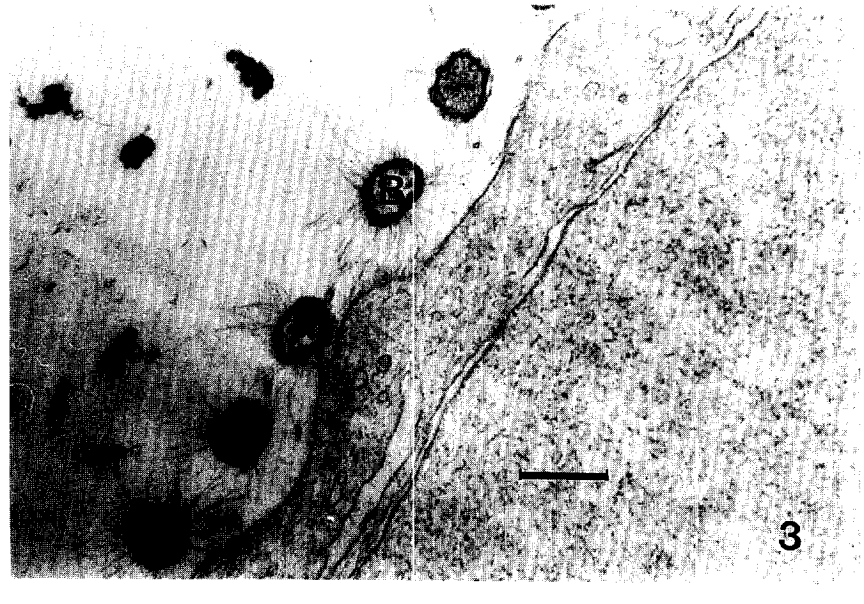

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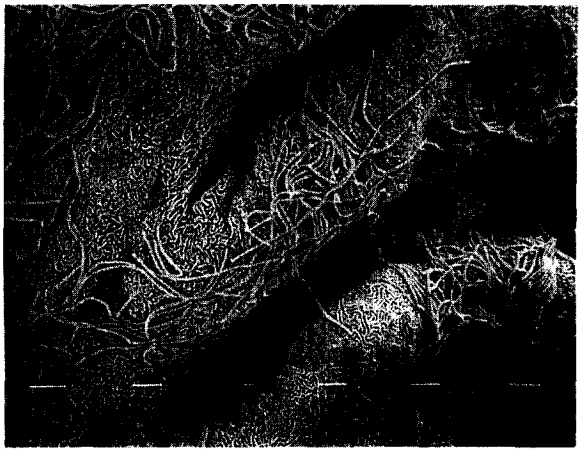

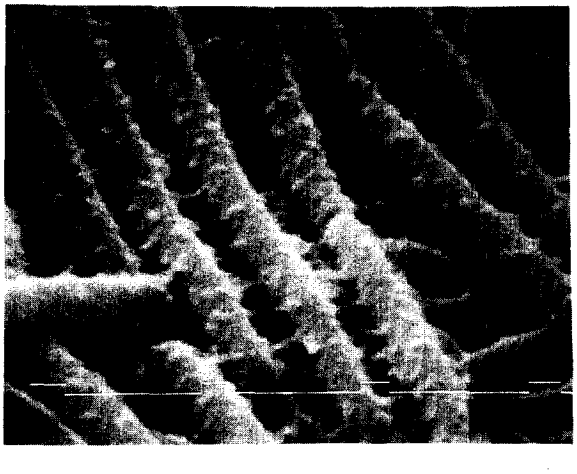

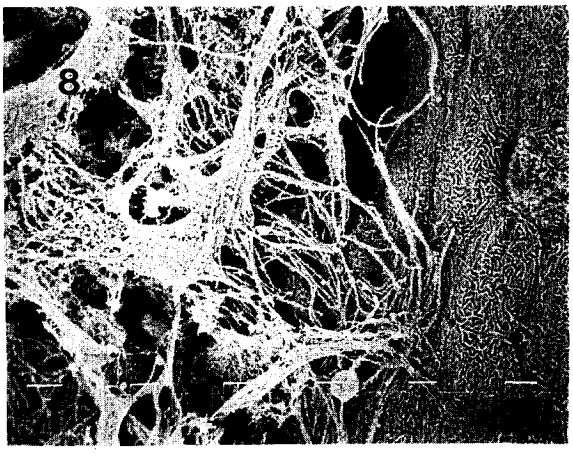

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