Short Communication

***N*-acyl Homoserine Lactone-Producing or -Degrading Bacteria Isolated from the Intestinal Microbial Flora of Ayu Fish (*Plecoglossus altivelis*)**

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(Received July 20, 2005—Accepted September 30, 2005)

A number of gram-negative fish pathogens have a quorum-sensing system. These bacteria produce *N*-acyl-L-homoserine lactone (AHL) as a quorum-sensing signal molecule. We isolated AHL-producing and degrading bacteria from the intestinal microbial flora of Ayu fish (*Plecoglossus altivelis*). AHL-producing and degrading isolates were identified as members of the genus *Aeromonas* and *Shewanella*, respectively. *Aeromonas* sp. strain MIB010 produced at least three kinds of AHLs and it was predicted that the production of extracellular protease in strain MIB010 was regulated through quorum sensing. *Shewanella* sp. strain MIB015 showed obvious AHL-degrading activity and interrupted the AHL-mediated production of protease by the *Aeromonas* sp. Intestinal *Shewanella* strains might be useful as probiotic bacteria effective in the prevention of bacterial infectious diseases controlled by quorum sensing.

Key words: quorum sensing, *N*-acyl-L-homoserine lactone, *Plecoglossus altivelis*, microbial flora

Quorum sensing is one of the cell-cell communication mechanisms based on cell population density in bacteria⁹. In many gram-negative bacteria, several kinds of *N*-acyl-L-homoserine lactone (AHL) have been identified as signal compounds involved in this mechanism and called autoinducers^{4,9}. AHLs are produced in bacteria, diffused outside and inside of bacteria, and regulate the expression of many of the genes responsible for bioluminescence, production of pigments or antibiotics and so on⁴. Notably, many gram-negative pathogens control the expression of virulence factors, the secretion of extracellular protease, pectinase and rhamnolipid, and the forming a biofilm⁴. Recently, AHL-degrading bacteria and enzymes have been reported. Genes encoding AHL lactonase, *aiiA* and *ahlD*, were cloned from *Bacillus* sp. strain 240B1 and *Arthrobacter* sp. strain IBN110, respectively^{6,15}. *Variovorax paradoxus* has been

reported to metabolize AHL as a sole carbon and nitrogen source using AHL acylase^{8,10}. *Arthrobacter* sp. strain IBN110 is able to control the virulence of *Erwinia carotovora* by reducing the AHL concentration and pectate lyase activity under the control of quorum sensing¹⁵. Transgenic plants expressing *AiiA* are resistant to the pathogen *E. carotovora*⁷.

Ayu fish (*Plecoglossus altivelis*) is commercially important for freshwater fisheries in Japan. In fish farms, bacterial infectious disease causes serious problems in ayu culture. Gram-negative pathogenic bacteria, such as *Aeromonas hydrophila*, *A. salmonicida* and *Vibrio anguillarum*, produce several kinds of AHLs^{3,18}. *A. hydrophila* and *A. salmonicida* produce two AHLs and regulate the production of extracellular protease or the formation of a biofilm through quorum sensing^{11,18}. *Vibrio anguillarum* produces three AHLs and regulates the expression of metalloprotease, the production of pigment and the forming of a biofilm through quorum sensing³. It was reported that several kinds of quorum-

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sensing inhibitors were effective in preventing the expression of the virulence factors of fish pathogens. Long chain AHL analogs, such as *N*-(3-oxododecanoyl)-L-homoserine lactone, reduce the activity of the extracellular protease in *A. hydrophila*¹⁹. To reduce mortality from vibriosis in rainbow trout, the effect of an inhibitor of quorum sensing, such as halogenated furanone, was investigated¹⁶.

A wide variety of bacteria are present in the microbial flora of fish intestines and they build symbiotic relationships in the fish intestines¹⁷. There has been no report about AHL-mediated quorum sensing systems in the fish intestinal symbiotic community. An analysis of AHL-producing and degrading mechanisms in the intestinal microbial flora might help us to elucidate how pathogens infect fish and understand the interaction between intestinal bacteria. In this study, we report the isolation of AHL-producing or degrading bacteria from ayu intestinal microbial flora. We also describe the identification of these bacteria and the regulation of the production of extracellular protease of AHL-producing bacteria using AHL-degrading bacteria.

To isolate the intestinal bacteria, the intestinal contents of cultured ayu fish (approximately 30 g) were suspended in 1 ml of phosphate-buffered saline (PBS) and diluted appropriately. These suspensions were spread on tryptic soy broth (TSB) plates with 1.5% agar. After incubation at 30°C, the colonies were streaked on new TSB plates. AHL-producing bacteria were isolated by cross streaking against *Chromobacterium violaceum* strain CV026 as the AHL biosensor¹⁸. Briefly, strain CV026 was streaked at the center of the TSB agar plate. The target bacteria were streaked on the same plate next to the CV026 line. If the target bacteria have AHL-producing ability, diffusible AHL produced by the target bacteria induces strain CV026 to produce a purple pigment. We chose three strains among the isolates, designated MIB007, MIB009 and MIB010, and determined their 16S rRNA gene sequences. Some intestinal bacteria showed obvious AHL-producing activity (Fig. 1A). The 16S rRNA coding region was amplified by PCR using previously described primers¹² and cloned into pGEM-T easy vector (Promega, Tokyo, Japan). Sequencing was performed using BigDye Terminator ver. 3.1 and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). DNA sequences were compared to others found in the DNA Data Bank of Japan using the on-line FASTA search engine (<http://www.ddbj.nig.ac.jp/>). All of the three strains were phylogenetically similar to members of the genus *Aeromonas* with *A. hydrophila* as their nearest neighbor (Fig. 2).

From a previous report, *A. hydrophila* and *A. salmonicida* produced *N*-butanoyl-L-homoserine lactone (BHL) and *N*-

Fig. 1. Identification and characterization of AHL-producing *Aeromonas* sp. strain MIB010. (A) Cross-streaks of *Aeromonas* sp. strain MIB010 against *C. violaceum* strain CV026. Production of diffusible AHL by *Aeromonas* sp. strain MIB010 induces strain CV026 to produce a violacein. (B) Analysis of AHLs produced by strain MIB010 using TLC. AHLs are visualized as pigments produced by strain CV026. Right lane (MIB010), concentrated cell-free supernatant of the strain MIB010 culture; left lane (M), synthetic standards BHL, HHL and OHL. (C) Extracellular protease activities in the culture supernatant with (squares) or without (circles) 10 μ M BHL. Samples were taken at various time points and analyzed for growth (absorbance at 600 nm, open symbols) and extracellular protease activity (azocasein units, solid symbols).

hexanoyl-L-homoserine lactone (HHL), and these AHLs stimulate the production of extracellular protease through quorum sensing¹⁸. We checked the structure of the AHLs by TLC¹⁴. Compared with the R_f value of standard AHLs, BHL, HHL and *N*-octanoyl-L-homoserine lactone (OHL), it was revealed that strain MIB010 produced at least three kinds of AHLs, BHL, HHL and an unknown AHL (Fig. 1B). It is commonly recognized that BHL is the major AHL and other AHLs are minor products between strain MIB010 and reported *A. hydrophila*¹⁸. The extracellular protease activity of strain MIB010 was measured with the azocasein assay¹. One azocasein unit was defined as the amount of en-

Fig. 2. Phylogenetic relationship of AHL-producing or degrading bacteria with closely related bacteria deduced from the sequence of 16S rRNA. The bacteria isolated in this study are shown in bold style. DDBJ/EMBL/GenBank accession numbers are given in parentheses. *Arthrobacter* sp. strain IBN110, *Variovorax paradoxus* strain VAI-C and *Bacillus* sp. strain 240B1 were used as previously reported AHL-degrading bacteria. The scale bar represents genetic distance (5 substitutions per 100 nucleotides).

zyme producing 0.01 A_{442} units per h. This protease activity increased in the early stationary phase and the increase was accelerated by adding exogenous BHL (Fig. 1C). These results indicated that AHL-mediated quorum sensing controlled the production of the extracellular protease in strain MIB010.

To screen for AHL-degrading bacteria in the intestinal community, a previously described protocol was modified slightly¹³. Briefly, the target strains were streaked at the center of the TSB agar plate. Strain CV026 was streaked to one side of the target strain. *Edwardsiella tarda* strain NUF251, which is a bacterium pathogenic to fish that produces two AHLs, was used as an AHL-positive strain¹⁴. Strain NUF251 was streaked on the other side of the target strain. The production of diffusible AHL by strain NUF251 induces strain CV026 to produce a purple pigment, but not if the target strain has the ability to degrade AHL. Among the intestinal bacteria, strains MIB011, MIB013 and MIB015 showed obvious AHL-degrading activity (Fig. 3A). In addition, these strains could also degrade AHLs diffusing from the intestinal nonpathogenic strain MIB010 (Fig. 3A). The 16S rRNA gene sequence of strain MIB015 showed high similarity with members of the genus *Shewanella* (Fig. 2). In a previous report, homoserine lactone-like molecules were detected in the culture supernatant of *Shewanella oneidensis* strain MR-1⁵. We checked for the existence of AHLs in the culture supernatant of these strains, but detected no AHL activity (data not shown). Though the AHL signal molecules are unstable at high pH (>7.5)², the pH of the culture supernatant of strain MIB015 was neutral at all times (data not shown). To estimate the

AHL-degrading activity, strain MIB015 and *Escherichia coli* strain MG1655 were cultured with 1 μ M HHL. The HHL remaining in the culture medium was extracted with ethyl acetate and detected using a strain CV026 indicator. The degradation of HHL in the medium is shown in Fig. 3B. Strain MG1655 as a negative control did not show obvious AHL-degrading activity, but strain MIB015 degraded HHL in the culture and almost all of the HHL was degraded after three days.

Fig. 3. Identification and characterization of AHL-degrading *Shewanella* sp. strain MIB015. (A) Degradation of AHL by *Shewanella* sp. strain MIB015. Degradation of AHL diffusing from the donor bacteria, *E. tarda* strain NUF251 and *Aeromonas* sp. strain MIB010, was determined by reduction of violacein production in strain CV026. (B) Degradation of HHL by *Shewanella* sp. strain MIB015 and *E. coli* strain MG1655 as a negative control. The concentration of HHL remaining was visualized using strain CV026.

Fig. 4. Effect of *Shewanella* sp. strain MIB015 on extracellular protease activity of *Aeromonas* sp. strain MIB010. Cell suspensions of strains MIB010 and MIB015 were mixed at a rate of 1:0 (solid squares), 1:1 (open circles) and 1:2 (open squares). The size of the halo area on a TSB-skim milk agar plate was measured at different time points as indicated.

We examined whether the AHL-degrading activity of *Shewanella* sp. strain MIB015 affects the extracellular protease activity of intestinal *Aeromonas* sp. strain MIB010. Overnight cultures of strain MIB010 and MIB015 were washed and resuspended in PBS. Both suspensions were diluted to an OD₆₀₀ of approximately 5.0. Then, 10 µl of the strain MIB010 suspension was mixed with 20 µl of PBS (control), 10 µl of PBS and 10 µl (1:1) or 20 µl (1:2) of the strain MIB015 suspension. Each mixture was spotted onto a TSB agar plate containing 2% skim milk. The size of the halo that developed was estimated everyday and is described in Fig. 4. The protease activity of strain MIB010 mixed with strain MIB015 was decreased compared with that of strain MIB010 alone, but there was no difference between the 1:1 and 1:2 mixtures. This result suggests that the degradation of AHL by strain MIB015 inhibits the expression of the extracellular protease via quorum sensing in strain MIB010. However, the mixture of strain MIB010 and strain MIB015 retained some extracellular protease activity. It was assumed that strain MIB010 produced a non-AHL-mediated extracellular protease as well as pathogenic *A. hydrophila*¹⁹⁾. The cell number of strain MIB015 mixed with strain MIB010 did not have an effect on the development of the halo. It was concluded that strain MIB015 depressed the expression of the AHL-mediated extracellular protease in strain MIB010 without interrupting cell growth.

In the case of rainbow trout, the dominant bacteria among the intestinal microbial flora were identified as members of the class *Gammaproteobacteria* including those of the genera *Aeromonas* and *Shewanella*¹⁷⁾. The genus *Aeromonas* is generally pathogenic to fish and produces opportunistic in-

fections. But, it was not elucidated whether all of the extracellular proteases secreted from the intestinal *Aeromonas* strains behave as a virulent factor in host fish. It was assumed that the intestinal AHL-degrading bacteria adjust the intestinal concentration of AHL and prevent the protease activity from reaching a lethal level. This is the first report that intestinal *Shewanella* strains have AHL-degrading activity and the suppression of AHL-mediated production of the extracellular protease by the intestinal *Aeromonas* strains. In the intestines, the decrease in the concentration of AHL by *Shewanella* strains might inhibit infection by pathogenic *Vibrio* or *Aeromonas*. Intestinal *Shewanella* strains might be useful as probiotic bacteria, which are effective in the prevention of bacterial infectious diseases.

Acknowledgements

We thank Hitoshi Kubota (Tochigi Prefectural Fisheries Experiment Station, Japan) for providing us with *Plecoglossus altivelis* and technical assistance.

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