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Distribution and Horizontal Transfer of Antibiotic Resistance Gene in Marine Environments

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Antibiotic-resistant bacteria distributed in fish farms are recently recognized as an agent that may give damages on human health. The bacteria indigenous to seawater environment may have become resistant to antibiotics in fish farms, or the bacteria which became resistant in terrestrial environments may have flown into fish farms. Both of the bacteria are likely to transfer the resistance genes to marine bacteria. It is our concern whether such resistant bacteria persist in seawater and disseminate drug-resistance gene to the other bacteria.

Key words : resistant bacteria, antibiotics, fish farm

Introduction

Antibiotic-resistant bacteria have increased in fish farm environment along with the use of antibiotics as chemotherapeutic agent for fishes. Although drug-free raising period is established for fishes before distribution in domestic market, the measure is only for decreasing the residual level of antibiotics in the fishes but not for the problem of drug resistant bacteria. The Antibiotic-resistant bacteria are now recognized not only as the agent compromising the effect of antibiotics in chemotherapy, but also as the possible agent transferring the resistance to human pathogenic bacteria. The question whether the drug-resistant bacteria are the threat to human health should be quickly answered. The article addressed at the scientific exchange meeting between National Fisheries University and Pukyong University is devoted to give some perspectives on this problem.

Antibiotic-Resistant Bacteria in Seawater Environment

Antibiotic usage in the fisheries sector of Japan totaled 179 metric tones in 2001. It was comprised of 38.2 tones of tetracycline, 41.4 tones of penicillin, and 99.4 tones of macrolide. The amount is comparable to the amount of clinical usage, 520 metric tones. The antibiotics are used only as chemotherapeutic agent but not as feed additives. Residual level of antibiotics in marine environment is reported to range from 10 to 0.1 μg/g in sediment, and seemed sufficient to induce the distribution of antibiotic resistant bacteria.

Antibiotic-resistant bacteria, becoming predominant under the selective pressure of antibiotics in clinical or stock-raising area, are transferred to seawater environments. Martinez-Urtaza have isolated drug resistant strains of *Salmonella enterica* serovar Typhimurium DT104 from mussels collected at a fish farm in Spain[10]. We also have isolated the strains of *Citrobacter* and *Salmonella* resistant to tetracycline and chloramphenicol from farmed fishes[5]. Interestingly, 6 strains of a nosocomial-infection agent, *Stenotrophomonas maltophilia*, isolated from cultured yellowtail, were resistant to penicillin, monobactam, panipenem and piperacillin but susceptible to cephem and ceftazidime[6].

It is most probable that the strains of *Stenotrophomonas maltophilia* seem to become resistant to panipenem in clinical environment, since the agent is permitted to use only for

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human, though the bacteria has been isolated from many natural different environments. The strains have been isolated from a fish farm where no antibiotic was used in the previous one year. As shown in Table 1, capability of the strains and a clinical strain of K279 to grow at a wide range of salinity seems to favor their distribution in seawater environment. It indicates that bacteria having a high capability to adapt to environment can survive in seawater environment even without selective pressure of antibiotics.

The process, in which *S. maltophilia* became drug-resistant, is elucidated by gene analysis. *S. maltophilia* is known to have L 1 metallo-β-lactamase and L 2 serine β-lactamase. Phylogenetic analyses with 16S rRNA gene and the genes of the two β-lactamases indicated that the 6 strains were divided into two clusters. The similarity between the cluster A and B was 89% for L 1 metallo-β-lactamase, while 74% for L 2 serine β-lactamase. Difference in the similarity suggested that at least one of the two enzyme genes was exogenously transferred from different bacteria. Given that L 1 metallo-β- lactamase originated outside cell, the resistance seemed to be obtained in clinical environment. Although some reports claimed that environmental strains of *S. maltophilia* are distinct from clinical strains of the species, the data suggested that the environmental strains had adapted to clinical environment by obtaining the gene of L 1 metallo-β-lactamase.

Photobacterium damsella, the causing-agent of pseudotuberculosis, is known as an example of marine bacteria that became drug-resistant under the selective pressure of antibiotic usage. Although the bacteria had been susceptible to antibiotics until 1977, the resistance to ampicillin was first reported in 1982, and 149 of 175 strains of *P. damsella*, isolated during the period from 1989 to 1991, showed resistance to either of 12 species of antibiotics including sulfamonomethoxine. According to the analysis by Kim and Aoki, many of the 149 strains showed a multi-drug resistance to kanamycin (KM), tetracycline (TC), chloramphenicol (CM), and sulfamonomethoxine (SA), and the strains showing resistance to KM, TC and SA amounted to the half of the resistant strains. Conjugation experiments with *Escherichia coli* as a recipient showed that the multi-drug resistances were conferred by the presence of transferable plasmids. Every plasmid for the multi-drug resistance to KM, TC and SA, the resistance to CM, KM, TC and SA and the resistance to KM and SA shared the DNA fragments of the same size in restriction analyses. Hence the resistances of *P. damsella* seem dependent on the same plasmid. The resistance to KM reaches 49% of the isolated strains[14]. Since KM is impermissible for use in Japan, distribution of the resistance should be caused by horizontal transfer of resistance gene assemble, either of which favored the distribution in seawater environment. KM resistance remained 63 to 85% of the isolated strains in the analyses in 2003 and 2004 by the Ministry of Agricultural, Forestry and Fisheries.

Distribution of Antibiotic Resistance Gene

The distribution of antibiotic resistance gene in marine environment is affected by antibiotic usage in fish farm and inflow from terrestrial environment. TC resistance in coastal area of Norway and Denmark, where no antibiotic usage had been reported, was mainly dependent on the gene of *tetE*. Although no relation to urbanization was found in the ratio of TC-resistant bacteria to total heterotrophic bacteria, *tetE*-containing bacteria were *Aeromonas hydrophila* not found in non-polluted area. Effect of urbanization was found in the distribution of *A. hydrophila*.

The determinants of TC-resistance were more frequently identified in fish farms with the history of antibiotic usage than was in the farm without it[5]. In fish farm A where chloramphenicol and erythromycin had been used, either of *tetB, C, D* was found in 11 of 21 TC-resistant bacteria. The determinants for the other 10 strains was not identified

Table 1. Specific growth rates (h^{-1}) of S. *maltophilia* strains.

Strain	NaCl concentration (%)			
	0	1	2	4
K279a (Clinical strain)	1.06	1.13	0.62	0.25
BL-15 (Environmental strain)	0.98	0.81	0.73	0.27

with PCR primers for *tetA, B, C, D, E, G, H, J* and *Y*. In fish farm B where chemotherapy had not been applied in the previous one year, no TC-resistance determinant was identified. In fish farm C where chemotherapy had been applied frequently, 23 of 27 TC-resistance strains contained *tetB*. Transferable plasmids like to enhance the distribution of TC-resistance in the sea where no usage of TC was recorded.

Genes of *tetB* in the 11 TC-resistance strains showed 99.8% homology to the *tetB* of a plasmid R100 found in *Escherichia coli*. A 100% homology was found between the bacteria of fish farm B and C. The genes of *tetC* found in 3 strains of fish farm C were 100% homologous to *tetC* in pRA3.2 of *Aeromonas salmonicida*, and 99.9% homologous to the *tetC* in pSC101 of *Salmonella* Typhimurium. *tetD* of a strain TC67 in fish farm C was 100% identical to *tetD* of *Shigella flexneri* Gibbon32055. *tetY* of strains TC72 and 73 were 100% homologous to *tetY* found in a IncQ-like plasmid pIE1120.

The distribution of TC-resistance determinants identical to those in clinical environment suggested the inflow of terrestrial resistant bacteria to seawater environment, whereas the analyses of 16S rRNA gene indicated that the resistant bacteria in the fish farm were included in the genus *Vibrio* and *Photobacterium* which were indigenous to seawater environment. Hence it is most probable that genes were transferred between marine and terrestrial bacteria. The presence of *Citrobacter* and *Salmonella* having *tetD* indicated the inflow from terrestrial environment, and the presence of identical *tetD* in *Alteromonas* indicated the transfer between marine and terrestrial bacteria. The low similarity, 96%, between marine and terrestrial bacteria suggested the low frequency of transfer of *tetG*. No isolation of *tetB* in *Aeromonas* indicated that the distribution of TC-resistant determinant were partly dependent on host strains.

There is still finding of many TC-determinants that were not identical to any of the known determinant. Nonaka and Suzuki[9] reported that an enzyme engaged in purine synthesis confer the resistance to TC. Morita[11], on the other hand, reported that *Vibrio parahaemolyticus* could efflux TC utilizing membrane potential developed by Na ion gradient.

Capability of resistant bacteria to transfer the resistant gene

Capability of resistant bacteria to transfer the resistance has been examined by the conjugation experiment with *Escherichia coli*. Eleven strains of 43 resistant strains, isolated in our experiment, transferred the TC resistance, together with Ampicillin- and CM-resisnatce[5]. The transconjugants also showed the resistance to minocycline and doxycycline, indicating that the genes were derived from clinical environment. Kim and Aoki also reported the capability of 147 strains of the previously denoted *P. damsella* to transfer the resistance to KM and SA. Sandaa et al[12] also reported that the strains of *Vibrio* and *Pseudomonas* could transfer oxytetracycline-resistance.

Gene Transfer in Marine Environment

Gene transfer in natural seawater environment seems to mainly occur in sediment, since the transfer frequency of 10^{-1} to 10^{0} was observed when the transfer was examined in the mixture of equal volume of seawater and sediment[13] and far higher than the frequency of 10^{-4} to 10^{-6} observed in a seawater experiment[4]. The higher frequency in the sediment is not only due to higher population density in the sediment, since the population in the seawater experiment was condensed ten folds, and the difference in the number of colony forming unit between seawater and sediment is not higher than thousand folds. Higher frequency also may be due to the presence of solid surfaces, because the surfaces are effective in condensing bacterial cells. The frequency in the sediment was enhanced 10-fold in the presence of antibiotics. And phenotypic examination indicated that a wide variety of bacteria could receive the resistance gene in sediment.

Gene transfer in natural seawater environment has been observed not only to culturable cells but also to the cells of viable but unculturable. Transfer to unculturable cells has been examined by using pBF 1 : *gfp*, the expression of *gfp* gene is regulated by a *lac* promoter and repressed in donor cells by a chromosomal insertion of a repressor *lacI*^{q}. The transfer of pBF 1 : *gfp* to recipient cells lacking the repressor results in the expression of *gfp* gene, and the recipient

cells were discernible by its fluorescence. Dahlberg et al, in an experiment with pBF 1 : *gfp*, calculated that the transfer frequency to the unculturable cells was hundred folds less than to culturable cells.

Our current activity

We have observed that an identical transferable plasmid was shared by marine bacterial isolates included in different genus. Fig. 1 shows large plasmid isolated from transconjugant strain. The structure analyses of the plasmids indicated the presence of transposon and integron that were layered over each other. The presence of identical plasmid in taxonomically different bacteria and the complicated structure of mobile element in the plasmid suggested the frequent horizontal transfer of gene in seawater environments. We think that we can track the transfer route of gene by analyzing the complicated structure, since the layering indicates the order of insertion.

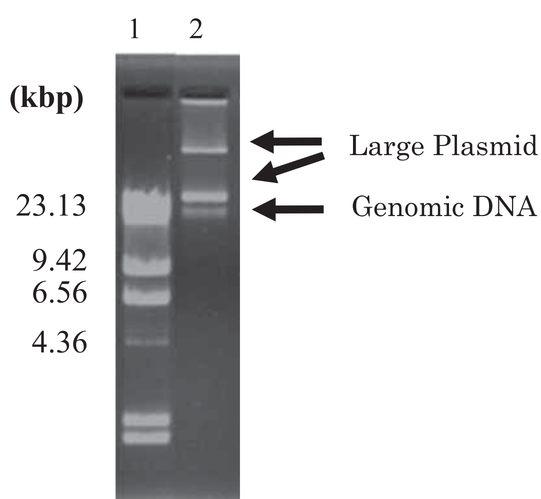

Fig. 1. Gel electrophoresis of large plasmids of a transconjugant strain. Lane 1, λ *Hin*dIII, DNA size marker; lane 2, E-TA 3.

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