Interdisciplinary Studies on Environmental Chemistry — Biological Responses to Contaminants, Eds., N. Hamamura, S. Suzuki, S. Mendo, C. M. Barroso, H. Iwata and S. Tanabe, pp. 1–8.

Tetracycline Resistance Gene in Asian Aquatic Environments

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(Received 22 January 2010; accepted 14 February 2010)

Abstract—Antibiotic pollution has recently been a global environmental and human health concern. When bacteria are exposed to antibiotics, they express drug resistance. Drug resistance genes can be vertically and horizontally transferred in the environment. In this paper, I provide a summary of our work on one category of tetracycline resistance gene, termed ribosomal protection protein gene. In particular, *tet*(M) is discussed in the context of its origin and distribution in aquatic environments of Asia.

Keywords: antibiotics, resistance, *tet*(M)

ANTIBIOTICS

Historically, the use of antimicrobial agents started in 1904 with the discovery of Tripan red by Ehrlich and Shiga. Ehrlich's team tested many color agents for chemotherapy, and found an active aromatic compound containing arsenic in 1910, which was the 606th compound in their trial; this is known as Salvalsan 606. In 1929 the antibiotic of penicillin was discovered by Fleming when his group found that the fungus *Penicilium notatum* produces a very selective inhibitor for *Staphylococcus* sp. Fleming's discovery showed that not only synthetic agents like Ehrlich's "magic bullet" but also a microbial product can be an effective antimicrobial drug. In 1943, Waksman started to use the word "antibiotics" when he discovered streptomycin. Antibiotics have thus been defined as a chemical substance derived from microorganisms, which have the capacity to inhibit growth, and even destroying other microorganisms in a dilute solution. After the initial age of discovery and since the 1970's many antimicrobial agents have been developed, although the discovery of new antibiotics has decreased in recent years. On this paper I use the word "antibiotic" to define those products derived from both natural and fully synthetic agents. Table 1 provides a summary of the four main categories of antibiotics based on their inhibitory target. Among the antibiotics, beta-lactams comprise the largest market share and many derivatives have been in development until now.

Tetracyclines (TC) are a broad-spectrum antibiotics produced by actinobacteria. TC inhibits protein synthesis of bacteria by preventing the

Table 1. Main classes of antibiotics.

Cell wall synthesis	Protein synthesis	DNA replication	Membrane
Bacitracin	Tetracyclines	Quinolones	Polymixins
Beta-lactams	Macrolides	Sulfonamides	
Glycopeptides	Aminoglycosides	Trimethoprim	
Fosfomycin	Chroramphenicol	Rifanpin	
	Fusidic acid	Coumarines	
	Ketolides		
	Lincosamides		
	Oxazolidinones		
	Streptgramins		

attachment of aminoacyl-tRNA to the ribosome, where the 30S subunit of bacterial ribosome is a target of TC. TCs are relatively cheap and their side effects are negligible, thus allowing for TC and its analogues to be broadly applied in fish, poultry and other agricultural industries. Although historically also applied to humans, their use is decreasing.

DRUG RESISTANCE IN BACTERIA

When bacteria contact with chemical substances, they show a positive or negative chemotaxis. If the substrates are acceptable for bacteria or can support bacterial growth, they show a positive chemotaxis and utilize the substrate as an organic source. If toxic, they respond by escaping from the chemical(s). Antibiotics selectively inhibit bacteria based on targeting a specific structure or function of bacteria, which means antibiotics act as toxins to bacteria. Mostly the targets of antibiotics are prokaryote-specific mechanisms and structures, which are not present in eukaryotes or they have different characteristics from those of eukaryotic cells. However, as shown in Table 2, bacteria inherently have potential drug resistance mechanisms or they can acquire exogenous genes conferring drug resistance. Drug resistance therefore occurs by such mechanisms. At present, four main categories of drug resistance mechanisms are known (Table 2). Some of these mechanisms have been well studied at the molecular level (Walsh, 2003). Many of the genes responsible for drug resistance can be horizontally transferred among the bacterial community. If pathogenic bacteria express the given drug resistance function, antibiotics become ineffective to control infectious diseases. In particular, the occurrence of multidrug resistance thus far has become a very big problem in human and animal clinical scenes. Understandably drug resistance in pathogenic bacteria constituted the majority of past research, however it is equally important to examine the full spectrum.

Our research group has been interested in the origin and dynamics of drug resistance genes in aquatic environments. Among the antibiotics, we have thus far reported on TC resistance; of environmental bacteria because of their capacity as a carriers and/or reservoir of the drug resistance. Some studies have reported

Table 2. Major categories of drug resistance in bacteria.

Alteration or synthesis of target	Impermeability by active efflux of the drugs	Production of an enzyme inactivating the drug	Impermeability by mutation in a porin channel
Penicillin binding proteins (PBPs) in MRSA	Resistance-nodule-cell division (RND)	Beta-lactamases	Loss of OprD
Mutation at quinolone resistance determining region (QRDR) in DNA gyrase and topo IV	ATP-binding cassette (ABC)	Aminoglycoside phosphotransferases (APHs)	Reduction of porins
	Multidrug and toxic chemical excrusion (MATE)		
	Small multidrug resistance pump (SMR)		
	Major facilitator superfamily (MFS)		

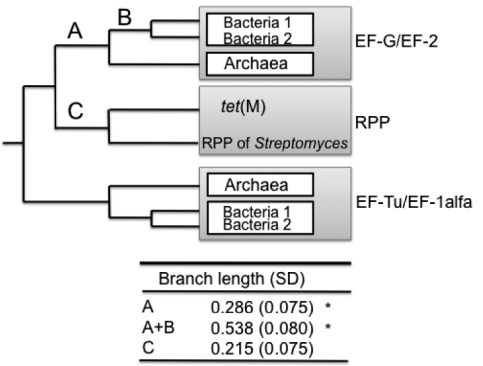

Fig. 1. Comparison of branch lengths between major nodes in the composite tee. * Indicates branch length was significantly longer than branch C ($p < 0.01$, paired t test); SD, standard deviation.

ecological findings of drug resistant bacteria and drug resistance genes in environmental bacteria (e.g. Aminov *et al*., 2001). This paper will summarize main topics on the origin and epidemiology of the *tet*(M) gene within the aquatic environment.

RIBOSOMAL PROTECTION PROTEIN (RPP) GENES

Roberts (2005) summarized series of TC resistance genes. At present, at least 40 genes have been known, among which the drug efflux and ribosomal protection protein (RPP) genes are the major mechanisms in antibiotic resistance. Of the TC resistance genes, we will focus on RPP genes particularly because of their unique property of being present across a wide distribution of taxonomically divergent bacteria (Connell *et al*., 2003), and being closely related to elongation factors, EF-G/EF-2 and EF-Tu/EF-1alfa (Sanchez-Pescador *et al*., 1988). Usually, drug resistance genes are "accessory" genes, which are not necessarily related to bacterial growth under natural conditions. However, the elongation factors are essential genes for growth and have a long evolutionary history from the superfamily of GTPase. Therefore, the study of RPP genes is a very effective and direct approach to understanding divergence in drug resistance (ribosome protection) and translation functions in bacteria.

Dr. Kobayashi who was an excellent postdoctoral researcher in my laboratory came up with the idea of making a composite phylogenetic tree of RPPs with elongation factor genes (Kobayashi *et al*., 2007b). His tree included two universal trees for EF-G/EF-2 and EF-Tu/EF-1alfa that formed clusters corresponding to their respective two protein groups from Bacteria, Archaea and Eukaryota. The RPPs branch formed an independent cluster at a point between the two EFs clusters. Branch lengths in the tree were compared as shown in Fig. 1. The branch length between the node for the RPP cluster and the primary divergence in this cluster (branch C) was shorter than that between the node for the RPP cluster and

the primary divergence in the EF-G/EF-2 cluster (branch A). Furthermore, branch C was less than half of branch A+B, indicating that the ancestor of the RPPs would have the extent function before divergence of the three superkingdoms. Taken together with data by Taylor and Raes (2004) showing that nonsynonymous substitution rate of genes generated by the duplication is accelerated, the RPP gene is generated by the duplication o the ancient GTPase gene. From Kobayashi's work, it can be concluded that RPP's extant function occurred prior to the appearance of streptomycetes which included TC producers. This suggests that RPPs evolved independently of the presence of antibiotics or TCs. RPP genes can be detected from natural pristine environments, which is explained by the previous evidences. At least one main function of RPPs should be protection of the ribosome from many chemical stressors including non-antibiotics.

DISTRIBUTION OF *tet*(M) IN VARIOUS AQUATIC ENVIRONMENTS

Among the RPPs, *tet*(M) has been known to a be broad host range gene and it can be detected by established PCR methods. Although TC efflux genes have been studied in the aquaculture environment (e.g. Furushita *et al*., 2003), RPPs have not been examined for their distribution in aquatic environments until early 2000. Dr. Kim who was a PhD student in my laboratory, screened isolated bacteria from Korean and Japanese coastal water, sediment and diseased fish (Kim *et al*., 2004). He found that *tet*(M) could be detected from Gram positive and negative bacteria including *Vibrio*, *Photobacterium*, *Lactococcus* and unknown Gram-positives. *Vibrio* species were major but various species were also present. The *tet*(M) possessing bacteria were isolated from fish intestine and seawater, suggesting that *tet*(M) is ubiquitously present in the aquaculture environment from these regions. Most fishermen yearly experienced the occurrence of oxytetracycline (OTC)-resistant bacteria just after administration of OTC to cultured fish; however, if drug administration is stopped, the OTC-resistant bacteria quickly disappear. The ubiquitous distribution of OTC resistance genes in environmental and fish intestinal bacteria may explain the quick response of bacteria to OTC. Dr. Nonaka who was a leader of our TC-resistance research team confirmed that exposure of OTC quickly accelerates the occurrence of OTC-resistant bacteria in Japanese aquaculture sites (Nonaka *et al*., 2007). The OTC resistance rate was less than 20% before OTC administration to fish, which dramatically increased to a rate of 53–61% immediately after drug treatment. *Vibrio* was the dominant genus of the OTC-resistant bacteria; however, other Gram-negatives and Gram-positives were also present. It is certain that drug therapy to fish is a form of selective pressure promoting increased numbers of resistant bacteria.

It is easy to understand the occurrence of OTC-resistant bacteria in aquaculture sites, because such environments are exposed to OTC. However, it is also known that drug resistant bacteria occur in the natural environment and even in pristine conditions (Gilliver *et al*., 1999). Previously we collected ocean sediments from the open Pacific Ocean, an environment far from anthropogenic sources of antibiotics or other pollutants. Dr. Nonaka and a postdoctoral researcher Dr.

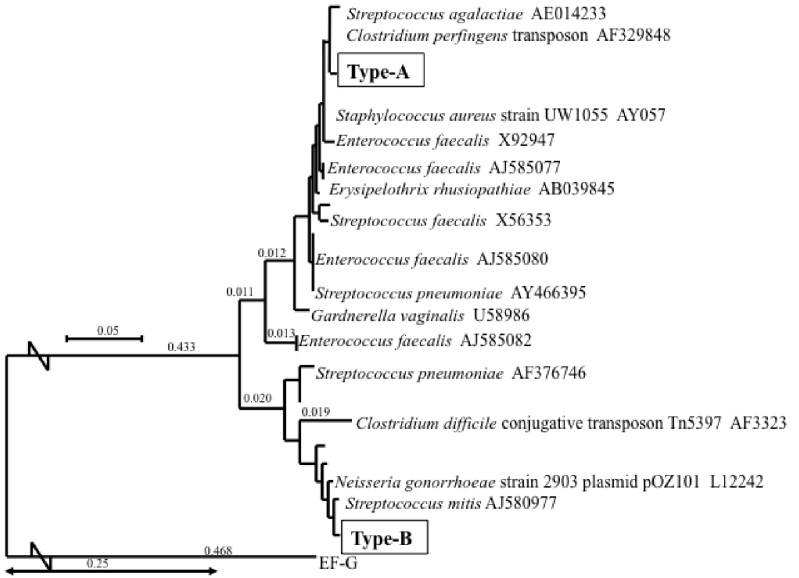

Fig. 2. Phylogenetic tree of *tet*(M) detected in OTC-resistant bacteria from sediment of open ocean.

Rahman analyzed *tet*(M) from OTC-resistant isolates and detected two different genotypes of *tet*(M) (Rahman *et al*., 2008b), as shown in Fig. 2. Genotype A and B were placed in genetically distant branches, suggesting different origins. The majority of *tet*(M) possessing isolates belonged to Bacillales (121 strains), followed by Actinomycetales (3 strains), Flavobacteriales (1 strain) and Pseudomonadales (1 strain). This indicated that *tet*(M) is present in various bacterial species in marine sediments, which is the natural reservoir of the *tet*(M) gene. We detected *tet*(M) from enteric bacteria of Antarctic Adélie Penguins (Rahman *et al*., 2008a), further suggesting that the natural environmental and animal enteric bacteria should have a wide range of RPP genes which are transferred among the bacterial community. Dr. Kobayashi's findings should be the strong evidence of this.

Also, in tropical aquatic environments from Asia, our team has found a diversity of *tet*(M) and related RPPs from Mekong River sediments (Kobayashi *et al*., 2007a). The RPP genes, *tet*(M), *tet*(S) and *tet*(W) were detected from a city canal and the main waterway of the Mekong River. Two genotypes of *tet*(M) were detected as similar to the ocean sediment case described above, however *tet*(S) and *tet*(W) were found to be of only one genotype. Again it is suggested that *tet*(M) should be derived from different origins and preserved by various bacteria in the environment.

To understand the relationship between TC/OTC-resistant bacteria and microbial community structure and diversity, we analyzed results from spatial

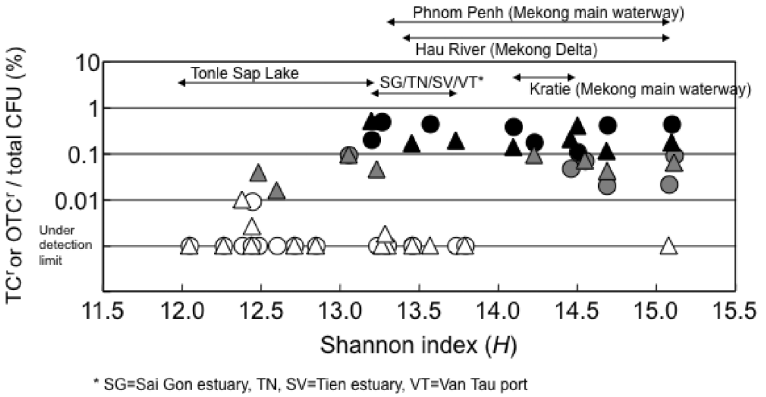

Fig. 3. Relationship between diversity index (*H*) and occurrence rate of TC/OTC-resistant bacteria (modified from Suzuki *et al*., 2008).

monitoring of the resistant bacteria in the region of Indochina, with particular focus on microbial diversity of the environment (Suzuki *et al*., 2008). The TC/OTC-resistant bacteria were detected with a high rate in the main waterway of the Mekong River whereas Tonle Sap Lake and the Saigon estuary showed lower rates. The Shannon index (*H*), an indicator of ecological diversity, was calculated from the denatured gradient gel electrophoresis (DGGE) profiles. Our data showed that the high diversity was positively correlated with the occurrence rate of TC/OTC-resistant bacteria (Fig. 3). As I mentioned before, RPP genes are shared between taxonomically diverse bacteria. The result of Fig. 3 suggests that the RPP genes are mixed in sediment and horizontal gene transfer occurs among various bacterial species within the natural environment. We found that *tet*(M) is transferred from marine *Vibrio* to *E. coli* at rate of 10^{-3} to 10^{-7}, and fish pathogenic *Lactococcus* to *Enterococcus faecalis* with a transfer rate of 10^{-5} to 10^{-6} (Neela *et al*., 2009). Mekong River sediments also expressed variations of the *tet*(M) gene, in which our hypothesis postulated that the origin of RPP genes divided prior to primary divergence of the three superkingdoms. Therefore, as well as horizontal gene transfer, a long evolutionary history might have produced different genotypes of *tet*(M) found in various environments.

Acknowledgments—I would thank the OTC-team in my laboratory, Drs. L. Nonaka, T. Kobayashi, S-R. Kim, H. M. Rahman and F. A. Neela, who contributed to this work. This work was partly supported by Grobal-COE program and Grants-in-Aid for Scientific Research from JSPS. I thank Dr. T. Miller for critical review of this manuscript.

REFERENCES

Aminov, R. I., N. Garrigues-Jeanjean and R. I. Mackie (2001): Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol*., **67**, 22–32.

Connell, S. R., D. M. Tracz, K. H. Nierhaus and D. E. Taylor (2003): Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob. Agents Chemother.*, **47**, 3675–3681.

Furushita, M., T. Shiba, T. Maeda, M. Yahata, A. Kaneoka, Y. Takahashi, K. Torii, T. Hasegawa and M. Ohta (2003): Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl. Environ. Microbiol.*, **69**, 5336–5342.

Gilliver, M. A., M. Bennett, M. Begon, S. M. Hazel and C. A. Hart (1999): Antibiotic resistance found in wild rodents. *Nature*, **401**, 233–234.

Kim, S. R., L. Nonaka and S. Suzuki (2004): Occurrence of tetracycline resistance genes *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiol. Lett.*, **237**, 147–156.

Kobayashi, T., F. Suehiro, B. C. Tuyen and S. Suzuki (2007a): Distribution and diversity of tetracycline resistance genes encoding ribosomal protection proteins in Mekong river sediments in Vietnam. *FEMS Microbiol. Ecol.*, **59**, 729–737.

Kobayashi, T., L. Nonaka, F. Maruyama and S. Suzuki (2007b): Molecular evidence for the ancient origin of the ribosomal protection protein that mediates tetracycline resistance in bacteria. *J. Mol. Evol.*, **66**, 228–235.

Neela, F. A., L. Nonaka, M. H. Rahman and S. Suzuki (2009): Transfer of the chromosomally encoded tetracycline resistance gene *tet*(M) from marine bacteria to *Escherichia coli* and *Enterococcus faecalis*. *World J. Microbiol. Biotechnol.*, **25**, 1095–1101.

Nonaka, L., K. Ikeno and S. Suzuki (2007): Distribution of tetracycline resistance gene, *tet*(M), in Gram-positive and Gram-negative bacteria isolated from sediment and seawater at a coastal aquaculture site in Japan. *Microbes Environ.*, **22**, 355–364.

Rahman, H. M., K. Q. Sakamoto, L. Nonaka and S. Suzuki (2008a): Occurrence and diversity of tetracycline resistance gene *tet*(M) in enteric bacteria of Antarctic Adélie penguin. *J. Antimicrob. Chemother.*, **62**, 627–628.

Rahman, H. M., L. Nonaka, R. Tago and S. Suzuki (2008b): Occurrence of two genotypes of tetracycline (TC) resistance gene *tet*(M) in the TC-resistant bacteria in marine sediments of Japan. *Environ. Sci. Technol.*, **42**, 5055–5061.

Roberts, M. C. (2005): Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.*, **245**, 195–203.

Sanchez-Pescador, R., J. T. Brown, M. Roberts and M. S. Urdea (1988): Homology of the TetM with translational elongation factors: implications for potential modes of tetM-conferred tetracycline resistance. *Nucl. Acids Res.*, **16**, 1218.

Suzuki, S., T. Kobayashi, F. Suehiro, B. C. Tuyen and T. S. Tana (2008): High occurrence rate of tetracycline (TC)-resistant bacteria and TC resistance genes relates to microbial diversity in sediment of Mekong river main waterway. *Microbes Environ.*, **23**, 149–152.

Taylor, J. S. and J. Raes (2004): Duplication and divergence: The evolution of new genes and old ideas. *Ann. Rev. Gen.*, **38**, 615–643.

Walsh, C. (2003): *Antibiotics—Action, Origin, Resistance—*. ASM Press, Washington, D.C.

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