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Examination of Integrated pML4 DNA Transfer from Genetically Modified Zebrafish to Bacteria

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The increased use of genetically modified (GM) organisms has raised concerns that the transgenes could be transferred to organisms in the wild. We examined the transfer of a foreign gene integrated into the chromosomal DNA of *rpsL* transgenic zebrafish to coexisting bacteria. This GM fish carries pML4 plasmid DNA containing a kanamycin (Km)-resistance gene as the foreign DNA. We isolated Km-resistant bacteria from feces, gut, and putrescent tissues and examined pML4 DNA transfer to them by colony direct PCR to amplify specific pML4 sequences. Km-resistant bacteria were grown from feces, but pML4 sequences were not detected in a stored sample of the colonies. We isolated 751 Km-resistant anaerobic bacteria from the gut, but none of them contained the pML4 sequence. To isolate Km-resistant bacteria from putrescent tissues, a GM fish euthanized on ice was incubated in rearing water at 28°C for 7 days. The number of Km-resistant bacteria increased with putrefaction, to about 10 times more at 7 days than that at day 0, but the pML4 sequence was not detected in approximately 900 colonies isolated from the putrescent tissues at day 7. These findings suggest that the Km-resistant bacteria isolated in this study were not produced by pML4 gene transfer from the GM fish.

Key words: transgenic fish, GMO, Km-resistant, gene transfer, bacteria

1. Introduction

Genetically modified (GM) organisms are increasingly generated for use as agricultural, bio-industrial, and food products. Their release into the environment has raised public concern about the potential risk of horizontal gene transfer, including the integrated gene transfer from GM organisms to bacteria in natural environments.

Gene transfer among bacteria occurs in natural environments[10,12]. Recombinant DNA, such as antibiotic resistance marker genes, is also transferred from GM bacteria to other bacteria in natural environments[8,18,20]. Since several kinds of GM plants useful for agriculture have been developed and commercialized, gene transfer from GM plants to bacteria in the field has been examined extensively as is seen elsewhere[17]. Occurrence of such events in the field has not been demonstrated so far[14], however, problems and difficulties have been pointed out in detecting such events with extremely low frequencies[7,14]. Occurrence of gene transfer from GM plants to bacteria in gut microbiota of animals has not been demonstrated, either[3,6,11,19].

Various kinds of GM fish have been developed, mainly as tools for biological and environmental research. GM fish have also been developed for food, although no commercially valuable GM food fish have been obtained yet[9]. To our knowledge, however, there are no reports dealing with gene transfer from GM fish to bacteria in fish flora and/or surrounding aquatic environments.

We previously developed GM *rpsL* zebrafish containing a shuttle vector plasmid (pML4) for detecting mutagens in water[1]. pML4 contains the *rpsL* gene as a target gene for detecting mutations and a kanamycin (Km)-resistance gene as a marker for transformation. Before we can use this *rpsL* GM zebrafish to monitor environmental mutagens outside the laboratory (*i.e.*, in the river), we have to clarify in advance the level of integrated DNA transfer from the GM fish to environmental bacteria. Therefore, we tried to determine the frequency of DNA transfer from GM fish to bacteria existing in or near the fish. Here, we showed that the amounts of pML4 DNA in environmental bacteria cultured from feces, gut, and putrescent tissues of GM fish were lower than the detection limit in all samples.

2. Materials and Methods

2.1. Fish and transgene

A GM zebrafish line carrying approximately 350 copies of the pML4 plasmid per haploid genome was established from the zebrafish AB line for a mutagenicity assay[1]. The GM fish have been maintained as hemizygous GM fish in rearing water (0.1% Instant Ocean salts, Tomita Pharmaceutical Co., Ltd. Tokushima, Japan) at 26°C over 10 gen-

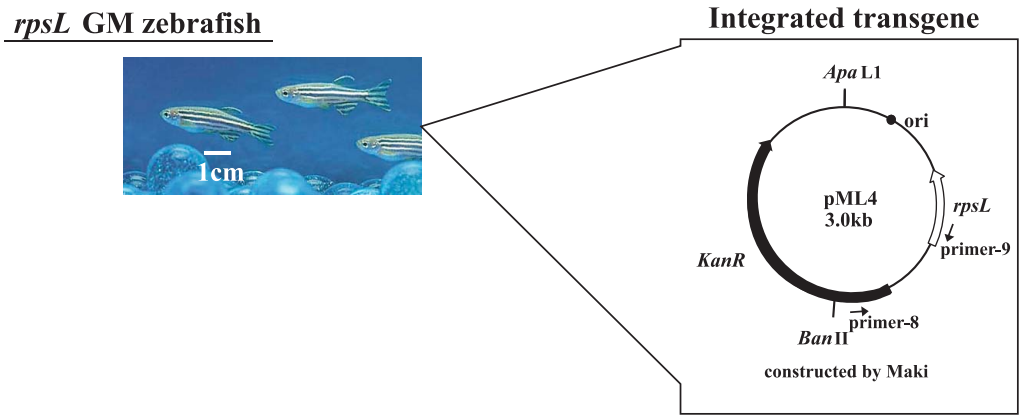

Fig. 1. Zebrafish and pML4 plasmid.

erations. The pML4 construct, which contains KanR, a Km-resistance gene[5], is shown in Fig. 1. The zebrafish AB line was used as non-GM control fish.

2.2. Isolation of bacteria and preparation of PCR samples

We used three kinds of media, Brain Heart Infusion (BHI; BD, NJ, USA), Sabouraud Dextrose Broth (SDB; BD), and Nutrient Broth (NB; BD), of which constituents and/or pH are different among them, for the cultivation of a wide variety of microorganisms according to the methods of Rawls *et al.*[15]

Feces collected from several GM fish and non-GM fish (17.8 mg and 4.7 mg wet weight, respectively) were suspended in BHI medium and spread onto BHI, SDB, or NB agar plates with or without Km (50 mg/l). Colonies were counted after the plates were aerobically cultured at 28°C for 3 days. Fifty colonies obtained from GM fish on Km-containing plates were randomly and individually picked for PCR. All the colonies from GM fish remaining on each Km plate type (BHI-Km, SDB-Km, or NB-Km) were scraped and combined into a pooled bacterial cell suspension in 10 mM Tris-HCl-1mM EDTA (TE) buffer (pH 8.0) for PCR; each pooled sample consisted of thousands of colonies grown on each type of plate.

A gut collected from a GM fish (19.4 mg wet weight) was immediately put into BHI medium, cut into pieces, and homogenized. The gut suspension was spread onto BHI, SDB, or NB plates with or without Km (50 mg/l) in an anaerobic globe box (Anaerorator model FEB-350, Sanki Kagaku Kougei Co., Ltd, Tokyo). These plates were cultured anaerobically in a BBL GasPak (BD) at 28°C for 3 days. A total of 751 colonies of Km-resistant bacteria were isolated individually. The 751 colonies were pooled into 76 bacterial cell suspensions containing 9 or 10 colonies each in TE buffer for PCR.

To obtain putrescent fish, a GM fish was killed by anesthesia on ice and placed in 50 ml rearing water and incubated at 28°C for 7 days. Just before the incubation, part of the abdomen (skin and intestine, 23.6 mg wet weight, Fig. 2a) was collected as control tissues (0 day), cut into pieces

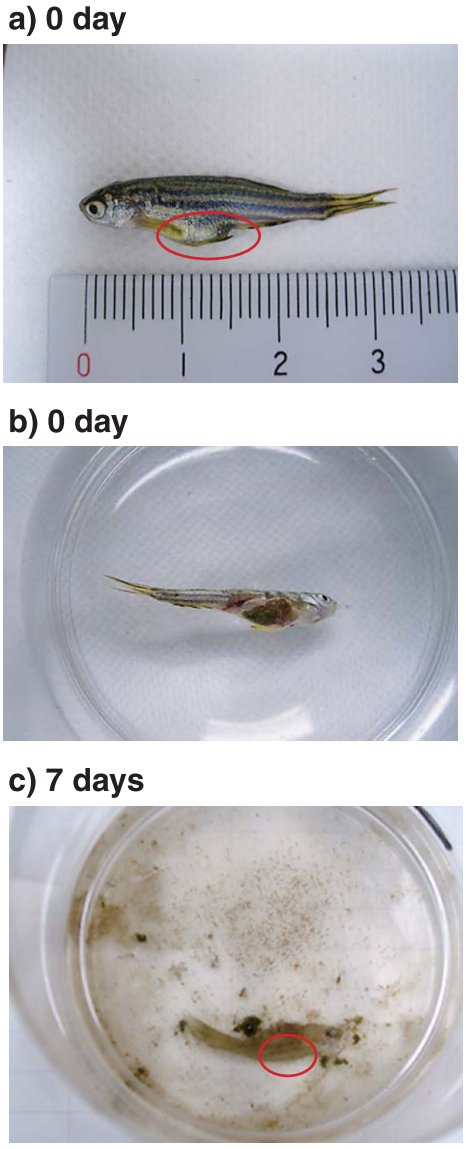

Fig. 2. Putrescence of euthanized GM zebrafish.
A GM fish euthanized on ice was placed in rearing water and incubated at 28°C for 7 days.
a, GM fish after death. Red circle indicates the part that was removed immediately as control tissues; b, GM fish at 0 days after removing control tissues; c, GM fish after 7 days incubation. Red circle indicates the part removed as putrescent tissue at 7 days.

and cultured immediately. At 7 days, 27 mg (wet weight) of putrescent tissue around the abdomen (Fig. 2c) was collected. Each tissue (before and after putrescence) was suspended in BHI medium and spread onto BHI, SDB, or NB plates with or without Km (50 mg/l). After the plates were aerobically cultured at 28°C for 3 days, approximately 300 Km-resistant colonies were picked from each plate (6 plates each of BHI-Km, SDB-Km, or NB-Km) cultured from control (0 day) or putrescent (7 days) tissues. The 300 colonies were pooled into samples containing 10 colonies each, to obtain approximately 30 bacterial cell suspensions in TE buffer for each kind of plate and PCR was performed.

2.3. Detection of pML4 sequence by PCR

Colony direct PCR was performed using primers specific for pML4; primer-8 (5'-ATCGCGAGCCCATTTATACC-3') and primer-9 (5'-GGCACGTTGCTTTTCGCAAC-3') (Fig. 1). We performed PCR in a final volume of 20 μl of Mg^{2+} plus *Ex Taq* buffer (Takara Bio Inc., Shiga, Japan) containing 0.2 mM dNTPs, 10 pmole of each primer, 0.5 unit of *Ex Taq* polymerase (Takara Bio Inc.) and 0.05 OD_{660} units of the bacterial cell suspension. The PCR conditions were 94°C for 2 min; 35 cycles of 94°C for 30 sec, 63°C for 30 sec, and 72°C for 1 min; and 72°C for 5 min. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. A detection limit for scraped and pooled samples from feces was estimated from an experiment employing, as a standard, *E. coli* RR1 transformed with pML4, and it was 2.6 bacterial cells per 20 μl of the PCR mixture (Fig. 3). A similar detection limit was also obtained by using another primer set specific for pML4. For colonies isolated from the gut and putrescent abdomen, 10 colonies were combined into one bacterial cell suspension for PCR, and therefore, the bacterial cell number from each isolated colony in the PCR mixture was much higher than the 2.6 cells/20 μl detection limit described above.

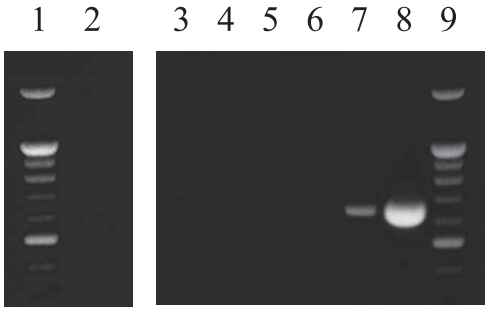

Fig. 3. Detection of pML4 DNA in bacterial samples prepared from feces by PCR.
Lanes 1 and 9, 100-bp ladders; lane 2, *E. coli* RR1 bearing no plasmid (negative control); lanes 3, 4, 5, mixture of colonies pooled from Km-BHI plates, SDB-Km plates, and NB-Km plates, respectively; lanes 6, 7, and 8, *E. coli* RR1 bearing the pML4 plasmid at 0.26, 2.6, and 2.6×10^6 cells/20 μL reaction mixture, respectively (positive control).

2.4. Calculation of pML4 DNA transfer frequency

The pML4 DNA transfer frequency in Km-resistant bacteria is calculated by the following formula:

(pML4 DNA transfer frequency in Km-resistant bacteria)=(Number of bacteria with transferred pML4 DNA)/(Number of Km-resistant bacteria checked by PCR)

The pML4 DNA transfer frequency in total bacteria cultured without Km was calculated by the following formula:

(pML4 DNA transfer frequency in total bacteria) ={pML4 DNA transfer frequency in Km-resistant bacteria ×Number of total bacteria (CFU/g dry sample)}/{Number of Km-resistant bacteria (CFU/g dry sample)}

3. Results

3.1. pML4 DNA transfer from GM fish feces to bacteria

Table 1 shows the number of bacteria cultured from feces collected from GM fish or non-GM fish using 3 kinds of medium. After 3 days of culture, approximately 10^7 to 10^9 CFU/g were grown on each plate without Km from both GM and non-GM fish. Considerable numbers of Km-resistant colonies grew from the feces of GM fish on BHI-Km and NB-Km plates (8.5×10^8 and 2.0×10^5 CFU/g dry feces, respectively), and these numbers were very similar to those cultured from feces of non-GM fish (8.5×10^8 and 2.1×10^5 CFU/g dry feces on BHI-Km and NB-Km plates, respectively). To examine whether the Km-resistant colonies origi-

Table 1. Km resistance and pML4 DNA transfer to bacteria from GM fish feces.

Medium		Total bacteria (CFU/g dry feces)	Km-resistant bacteria (CFU/g dry feces)	Km resistance rate	Number of bacteria used for PCR	Number of pML4 DNA transferred bacteria	pML4 DNA transfer frequency in total bacteria
BHI	GM	2.3×10^9	8.5×10^8	3.7×10^{-1}	59645	<2.6	$<1.6\times10^{-5}$
	Non-GM	1.8×10^9	8.5×10^8	4.7×10^{-1}	n.d.	n.d.	n.d.
SDB	GM	2.2×10^8	1.5×10^7	6.8×10^{-2}	7677	<2.6	$<2.3\times10^{-5}$
	Non-GM	4.4×10^7	6.4×10^5	1.5×10^{-2}	n.d.	n.d.	n.d.
NB	GM	2.5×10^9	2.0×10^5	8.0×10^{-5}	2879	<2.6	$<7.2\times10^{-8}$
	Non-GM	1.6×10^9	2.1×10^5	1.3×10^{-4}	n.d.	n.d.	n.d.

n.d.: not determined.
The detection limit was estimated to be 2.6 bacterial cell per 20 μL for PCR of scraped and pooled samples. Therefore, the pML4 transfer frequency in Km-resistant bacteria used for PCR was calculated to be below 2.6 cells/number of Km-resistant bacteria used for PCR, and the pML4 transfer frequency in total bacteria was calculated to be below 2.6 cells/number of Km-resistant bacteria for PCR × Km-resistance rate. (For example, for the BHI plate, the value was calculated to be below $2.6/59645\times3.7\times10^{-1}=1.6\times10^{-5}$.)

nating from GM fish contained pML4 DNA, we randomly isolated 50 colonies each grown from GM fish feces on the BHI, SDB, and NB plates. All the remaining colonies on each plate were scraped and combined with colonies from other plates containing the same medium into 3 pooled samples and subjected to PCR along with individually isolated colonies. After gel electrophoresis analysis, no PCR products were detected in the 50 individual colonies or in the pooled samples (Fig. 3). The pML4 DNA transfer frequency in the 3 pooled samples was calculated to be below 10^{-5}–10^{-8} (Table 1).

3.2. pML4 DNA transfer from GM fish gut to anaerobic bacteria

Anaerobic intestinal bacteria from GM fish gut were cultured on BHI, SDB, or NB plates with or without Km (Table 2). Substantial numbers of Km-resistant bacteria were detected only on BHI-Km plates. The Km-resistance rate of bacteria cultured on BHI plates was 8.7×10^{-3} as calculated from the number of Km-resistant colonies (9.6×10^{4} CFU/g dry gut) divided by the total number of colonies (1.1×10^{7} CFU/g dry gut). We did not scrape together all the colonies on the plates, but isolated 751 colonies individually to avoid contamination of GM fish tissues in the PCR samples. We looked for pML4 DNA in the 751 isolated Km-resistant intestinal bacteria by PCR but did not detect any PCR products corresponding to pML4 DNA, indicating that pML4 was transferred to less than one out of 751 isolated bacteria. From these results, we estimated the pML4 transfer frequency to be under 1.2×10^{-5} ($1.2\times10^{-5}=1/751\times8.7\times10^{-3}$).

3.3. pML4 DNA transfer from putrescent GM fish tissue to bacteria

We next examined whether the gene could be transferred from decomposing fish tissue. After the fish was killed by exposure to cold, we removed a part of abdomen containing skin and gut as day 0 control tissues (Fig. 2a, b). The remainder of the fish was incubated in rearing water at 28°C. After 5 days of incubation, the outlines of the dead fish became unclear, likely due to the putrescence of the tissues. On day 7, we collected some of this tissue from around the abdomen (Fig. 2c). Both control and putrescent tissues were cultured aerobically on BHI, SDB, and NB plates. Considerable numbers of Km-resistant bacteria grew from the control tissue harvested on day 0, but after 7 days incubation, the numbers of Km-resistant bacteria increased to 7.4–13.3 times that at day 0 (Table 3, Fig. 4). We isolated approximately 300 colonies from each plate, and PCR was performed as described in the Materials and Methods. No amplified products were detected in any of the bacterial samples from BHI-Km, SDB-Km, or NB-Km plates at either 0 and 7 days, indicating that pML4 DNA transfer frequencies were below 10^{-3}.

3.4. DNA sequence homology to Km-resistance genes deposited in GenBank

Considerable numbers of Km-resistant bacteria were cultured even from feces of non-GM fish (Table 1). Km is an aminoglycoside antibiotic and resistance to Km is often due to enzymatic inactivation of Km. The DNA sequences of many bacterial genes encoding aminoglycoside-modifying enzymes that confer Km-resistance have been already determined (Table 4).

We performed a homology search between KanR in pML4 and all of the Km-resistance genes we found in the DNA Data Bank of Japan and in the review of Shaw *et al.*[16] (Table 4), and found only very low homologies between KanR and other bacterial genes, except for the uncultured eubacterium plasmid pIE1130 (99.1% homology) and

Table 2. Km resistance and pML4 transfer to bacteria from GM fish gut.

Medium	Total bacteria (CFU/g dry gut)	Km-resistant bacteria (CFU /g dry gut)	Km resistance rate	Number of isolated bacteria used for PCR	Number pML4 DNA transferred bacteria	pML4 DNA transfer frequency in total bacteria
BHI	1.1×10^{7}	9.6×10^{4}	8.7×10^{-3}	751	<1	$<1.2\times10^{-5}$
SDB	1.9×10^{5}	0	n.d.	n.d.	n.d.	ND
NB	5.4×10^{7}	0	n.d.	n.d.	n.d.	ND

ND: Not detected, n.d.: not determined.

Table 3. Km-resistant bacteria and pML4 transfer to bacteria from dead GM fish.

Medium	Culture days	Total bacteria (CFU /g dry tissue)	Km-resistant bacteria (CFU /g dry tissue)	Km resistance rate	Number of isolated bacteria used for PCR	Number of pML4 DNA transferred bacteria	pML4 DNA transfer frequency in total bacteria
BHI	0	6.2×10^{9}	1.8×10^{7}	2.9×10^{-3}	310	<1	$<9.5\times10^{-6}$
	7	3.5×10^{9}	2.4×10^{8}	6.9×10^{-2}	310	<1	$<2.2\times10^{-4}$
SDB	0	3.6×10^{7}	2.7×10^{7}	7.5×10^{-1}	310	<1	$<2.4\times10^{-3}$
	7	2.7×10^{8}	2.0×10^{8}	7.4×10^{-1}	310	<1	$<2.4\times10^{-3}$
NB	0	2.0×10^{9}	4.1×10^{6}	2.0×10^{-3}	262	<1	$<7.9\times10^{-6}$
	7	2.4×10^{9}	4.8×10^{7}	2.0×10^{-2}	302	<1	$<6.5\times10^{-5}$

Klebsiella pneumoniae (72.2% homology).

4. Discussion

As a first trial for investigating gene transfer from GM fish to environmental bacteria, we cultured Km-resistant bacteria from feces, gut, and putrescent tissues of GM fish and examined by PCR whether they contained pML4 DNA sequences. Appreciable numbers of Km-resistant bacteria were cultured in all samples, but the pML4 sequences in them were below the detection limit, suggesting that these Km-resistant bacteria were not generated by pML4 DNA transfer from the GM fish. The pML4 DNA transfer frequencies were calculated to be below 10^{-3} to 10^{-8} (Tables 1–3). Notably, similar numbers of total and Km-resistant bacteria were grown from GM and non-GM feces and similar frequencies of Km resistance were observed (Table 1), suggesting that these Km-resistant bacteria are probably indigenous to natural environments. These findings are consistent with the existence of many Km-resistant bacteria

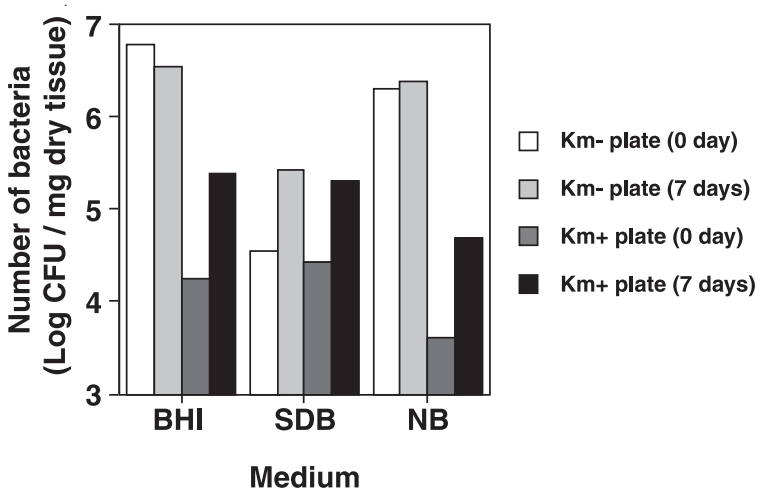

Fig. 4. CFU of total and Km-resistant bacteria isolated from euthanized GM zebrafish before (day 0) and after (day 7) putrescence.

Table 4. Km resistance genes in bacteria.

Km-resistant bacteria	Enzyme[a] (gene)	DDBJ/EMBL/GenBank accession no.
Escherichia coli	(Kanamycin resistance transposon Tn903; KanR)[b]	V00359
Campylobacter coli plasmid pIP1433	Kanamycin resistance protein (*aph*A-3)	M26832
Pseudomonas aeruginosa	Aminoglycoside acetyltransferase (*aac(6')-Iae*)	AB104852
Staphylococcus aureus	Kanamycin nucleotidyltransferase (*aad*D)	AF181950
Streptomyces albulus	Kanamycin acetyltransferase (*kat*)	AB116646
Streptomyces kanamyceticus	16S rRNA methylase *(kmr*)	AB164229
Streptomyces kanamyceticus	Aminoglycoside 6'-N-acetyltransferase (*kac*)	AB164230
Uncultured eubacterium plasmid pIE1130	Aminoglycoside phosphotransferase *(aph(3')-I*)	AJ271879
Vibrio cholerae	Aminoglycoside adenylyltransferase (*aadB*)	AF221902
Pseudomonas aeruginosa PST-1[c]	Aminoglycoside-(3)-N-acetyltransferase (*aac(3)-IIIa*)	X55652
Pseudomonas aeruginosa[c]	n.d.[d] (*aac(3)-IIIb*)	L06160
Pseudomonas aeruginosa[c]	Aminoglycoside 3'-N-acetyltransferase (*aac(3)-IIIc*)	L06161
Unidentified bacterium[c]	Aminoglycoside adenylyltransferase (*ant(2'')-Ia*)	X04555
Escherichia coli plasmid RP4[c]	Aminoglycoside-3'-phosphotransferase (*aph(3')-Ib*)	M20305
Klebsiella pneumoniae[c]	Aminoglycoside-3',5''-phosphotransferase-I (*aph(3')-Ic*)	M37910
Escherichia coli[c]	Neomycin phosphotransferase (*aph(3')-IIa*)	V00618
Streptococcus faecalis[c]	3',5''-Aminoglycoside phosphotransferase type III (*aph(3')-IIIa*)	V01547
Acinetobacter baumannii[c]	3'-Aminoglycoside phosphotransferase (*aph(3')-VIa*)	X07753
Campylobacter jejuni[c]	Kanamycin phosphotransferase (*aph(3')-VIIa*)	M29953

[a], Registered name in DDBJ/GenBank.
[b], The transgene integrated in the GM fish used in this study carries this gene, KanR.
[c], Reported in the review of Shaw *et al.*[16]
[d], n.d. not described in Shaw *et al.*[16]

confirmed by the survey of the previously identified bacteria; almost all Km-resistant bacterial genes listed (Table 4) have no homology with KanR in pML4. Boon reported that a considerable percentage of bacteria isolated from river water in rural, semi-rural, and urban sites show resistance to several antibiotics, including Km[2].

The variation in the DNA transfer frequency (below 10^{-3} to 10^{-8}) depended on the percentage of Km-resistant bacteria among the total cultured bacteria and the number of colonies isolated for PCR. To obtain more precise data, we may need to isolate much higher numbers of Km-resistant colonies for PCR, for example, from putrescent tissues cultured in SDB medium (Table 3). The transformation frequencies of naturally transformable bacteria were reported to be 10^{-2} to 10^{-9} [12], and the transformation frequencies of a bacterial strain specialized to receive a GM plant transgene were at the 10^{-8} level[13]. Considering these reports and the non-conjugative nature of the pML4 plasmid, the frequency of transformation of the bacteria surrounding GM fish by pML4 might be lower than natural bacterial transformation and GM plant DNA transfer. To detect such rare events using the strategy employed here, we might need to isolate much higher numbers of Km-resistant colonies and to culture them to create much more bacterial colonies. This strategy, however, contains serious problems; bacteria receiving pML4 may not always become Km-resistant because the Km-resistance gene in pML4 may not always be expressed in a heterologous bacterial host. Moreover, only a small proportion of the bacteria present in the feces, gut, and fish environment are culturable. To estimate more precisely the possibility of DNA transfer from GM fish to surrounding bacteria, new strategies might be required, such as the recently developed gnotobiotic zebrafish technique[15], and/or the establishment of a new transgenic fish line bearing a marker gene that is efficiently selectable and functional in a broad range of bacteria.

Despite the limitations to the experiments described above, we showed at least that an integrated transgene of GM fish, pML4, did not cause transfer of culturable Km resistance to bacteria existing with the GM fish under the experimental conditions, suggesting that using *rpsL* GM fish[1] for monitoring aquatic environments is unlikely to cause a serious problem with pML4 transfer.

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