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Short Communication

Distribution and characteristics of plasmid mobilizers in aquatic and soil environments and activated sludge

Daisuke Inoue,[1,*] Katsushi Murashige,[1] Kazunari Sei,[1] Satoshi Soda,[1]
Michihiko Ike,[1] and Masanori Fujita[1,2]

[1] *Division of Sustainable Energy and Environmental Engineering, Osaka University, Osaka 565–0871, Japan*
[2] *Kochi National College of Technology, Kochi 783–8508, Japan*

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Recent advances in biotechnology have led to the development of genetically engineered microorganisms (GEMs) with potential agricultural, environmental and industrial applications. However, the environmental release of GEMs has potential adverse effects on the public health and structure and/or function of natural ecosystems. These potential risks are related to the dispersal and expression of the recombinant DNA (Morrissey et al., 2002). Therefore, for risk assessment related to the use of GEMs, knowledge on the transferability of recombinant DNA in the environment is needed.

Conjugative functions such as *tra* and *mob* genes are usually deleted from recombinant plasmids in order to limit their dispersal in the environment. Nevertheless, transfer can occur via conjugation-mediated mobilization or cointegration if the host of nonconjugative mobilizable (Tra^- Mob^+) or nonmobilizable (Tra^- Mob^-) plasmids receives a conjugative plasmid from indigenous bacteria (Davison, 1999). Therefore, the transfer of recombinant plasmids in the environment would considerably depend on the distribution and characteristics of mobilizers bearing conjugative plasmids. Although several studies have been made on the isolation of bacteria with conjugative plasmids from environmental samples, few have compared the distribution and characteristics of plasmid mobilizers in various environments (Top et al., 1994).

Recently, Disqué-Kochem et al. (2001) designed universal PCR primers to detect the *traG*- and *trbB*-like genes in the *tra* region, and demonstrated that they could detect bacteria with plasmids capable of mobilizing a Tra^- plasmid from an agricultural soil sample. Their detection system is useful for screening mobilizers from various environmental samples because of the high sensitivity of PCR and wide detection range of their universal primers. Here, we screened putative mobilizers from aquatic and soil environments and activated sludge using their PCR-based detection system, and qualitatively assessed the distribution of mobilizers in the environment. In addition, we taxonomically classified isolated mobilizers and determined the approximate sizes and the incompatibility (Inc) groups of their plasmids.

Aquatic, activated sludge and soil samples were subjected to the screening of mobilizers. Nine aquatic samples were collected from rivers, lake and ponds in Hyogo, Osaka or Shiga. Four activated sludge samples were obtained from 2 municipal wastewater treatment plants in Osaka and Hyogo. Ten soil samples

* Address reprint requests to: Dr. Daisuke Inoue, Division of Sustainable Energy and Environmental Engineering, Osaka University, 2–1 Yamadaoka, Suita, Osaka 565–0871, Japan.
Tel: +81–(0)6–6879–7674 Fax: +81–(0)6–6879–7675
E-mail: inoue@wb.see.eng.osaka-u.ac.jp

were taken from a 0–30 cm depth of agricultural and forest fields in Fukuoka, Kagoshima, Kumamoto, Osaka, Yamaguchi or Yamanashi. In addition, 2 soil samples were obtained from oil-contaminated sites. Predominant heterotrophic bacteria were first obtained from aquatic, activated sludge and soil samples using Yoshikura 0.05 medium (Soda et al., 1998) or 1/10 CGY medium (Pike et al., 1972), CGY medium (Pike et al., 1972) and PYG medium (Soda et al., 1998) or R2A medium, respectively. Then, colonies used for screening mobilizers were randomly chosen considering their morphology.

Screening of mobilizers was carried out by colony PCR (Joshi et al., 1991) targeting *trbB*-like DNA sequences by using a universal primer set [B-1, B-2] (Disqué-Kochem et al., 2001). PCR amplifications were performed by the thermal profile of Disqué-Kochem et al. (2001) in a Mastercycler Standard (Eppendorf). Aliquots (5 μl) of PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with 0.5 μg/ml of ethidium bromide solution. A colony which showed the fragment of anticipated size (ca. 370 bp) was defined as a putative mobilizer.

Isolated putative mobilizers were taxonomically classified using a diagnostic table of bacteria (Cowan and Steel, 1974) and API20NE (BioMerieux Japan). Plasmid DNA of isolated putative mobilizers was extracted by a slightly modified method of Birnboim and Doly (1979). Aliquots (5 μl) of extracted plasmid DNA were electrophoresed on a 0.6% agarose gel stained with 0.5 μg/ml of ethidium bromide solution, and the plasmid size was measured. If needed, plasmid DNA was digested with the restriction enzyme *Sac*I or *Kpn*I (TaKaRa). Plasmid DNA of mobilizers was also subjected to PCR amplification specific for plasmids belonging to the Inc groups IncP, IncN, IncW and IncA/C to assign to known Inc groups. PCR amplification of partial sequences of IncP, IncN, IncW and IncA/C plasmids was performed using the primers designed previously (Götz et al., 1996; Llanes et al., 1996) according to the thermal profile of Götz et al. (1996) with the annealing temperature of 57, 55, 58 and 51°C, respectively.

Table 1 shows the proportion of colonies with the *trb*B-like DNA sequences, i.e. putative mobilizers, among culturable heterotrophic bacteria in various environments. Putative mobilizers were detected in all environments: aquatic, soil, and activated sludge. Among a total of 542 bacterial colonies, 9 colonies (1.7%) were determined as putative mobilizers. Putative mobilizers were detected in nearly half of the samples obtained from both aquatic environments and activated sludge. In contrast, among 12 soil samples, a putative mobilizer was detected in only one forest soil sample obtained in Yamaguchi Prefecture. Contrary to our expectations, no putative mobilizer was detected in oil-contaminated soil samples. These results suggest that plasmid mobilizers would be locally predominant in the environment, and that the localization would be higher in soil environments than in aquatic environments or activated sludge.

Taxonomical classification showed that all but *Moraxella* sp. MR-6 belonged to pseudomonads irrespective of their source (Table 2). This suggests that pseudomonads may be one of the representative mobilizer groups in the environment.

Plasmids with approximately 100 kb were observed in some putative mobilizer strains (Table 2). On the other hand, in *Chryseomonas luteola* IU-7, the visible plasmids were smaller than the 30 kb needed for encoding the conjugative function (Rochelle et al., 1989). In this strain, plasmids with the conjugative function may be present on non-visible plasmids or megaplasmids (Disqué-Kochem et al., 2001), which are difficult to detect by the method employed in this study.

PCR amplification with primers specific for several Inc groups revealed that all the putative mobilizers tested had IncP plasmids (Table 2). In addition, *C. luteola* IU-7, *Ochrobactrum anthropi* MR-18 and *Sphingomonas paucimobilis* YF-1 made fragments of the anticipated sizes specific for IncN, IncA/C and IncN, W and A/C plasmids in PCR, respectively (Table 2). Although two plasmids were observed in *S. paucimobilis* IM-2, an amplification product was detected only in IncP-specific PCR. The other plasmid in this strain would belong to the Inc group which were not examined in the present study. Götz et al. (1996) indicated that IncP plasmids were more prevalent than other broad-host-range conjugative Inc group plasmids such as IncN and IncW plasmids in soil samples. Other studies have shown that conjugative plasmids isolated from soil and activated sludge samples mainly belonged to IncP (Dröge et al., 2000; Top et al., 1994). Therefore, it is suggested that IncP plasmids may be the representative mobilizing plasmids in the environment.

Our results suggest the local predominance of plasmid mobilizers in the environment. This implies that

Table 1. Relative abundance of bacteria bearing the *trbB*-like DNA sequences in the environment.

Environment	Sample source	Sampling location	No. sample	No. colonies tested	No. putative mobilizer	% putative mobilizer
Aquatic	Ina River	Hyogo, Osaka	3	123	3	2.4
	Yodo River	Kyoto, Osaka	2	55	1	1.8
	Kizu River	Kyoto	1	19	0	0
	Lake Biwa	Shiga	1	25	1	4.0
	Zuion Pond	Osaka	1	20	0	0
	Inukai Pond	Osaka	1	21	0	0
	Total		9	263	5	1.9
Activated sludge	Plant A	Osaka	3[a]	51	3	5.9
	Plant B	Osaka	1	17	0	0
	Total		4	68	3	4.4
Soil	Forest soil	Fukuoka	1	17	0	0
		Kagoshima	1	15	0	0
		Kumamoto	2	31	0	0
		Osaka	1	23	0	0
		Yamaguchi	1	11	1	9.1
		Yamanashi	1	13	0	0
	Agricultural soil	Kumamoto	1	22	0	0
		Osaka	2	53	0	0
	Oil-contaminated soil	Kumamoto	2	26	0	0
	Total		12	211	1	0.47
Total			25	542	9	1.7

[a] Activated sludge samples in plant A were obtained on different dates or from different treatment processes.

Table 2. Characteristics of isolated mobilizers.

Environment	Strain	Sample source (Sampling location)	Taxonomical classification	Approximate plasmid size (kb)	Inc group
Aquatic	IM-2	Ina River (Osaka)	*Sphingomonas paucimobilis*	95, 25	P
	IM-48	Ina River (Osaka)	*S. paucimobilis*	—[b]	P
	IU-7	Ina River (Osaka)	*Chryseomonas luteola*	15	P, N
	LB-15	Lake Biwa (Shiga)	*Brevundimonas vesicularis*	—	P
	RU-33	Yodo River (Kyoto)	*B. vesicularis*	—	P
Activated sludge	M1-9	Plant A (Osaka)	Loss[a]	—	—
	MR-6	Plant A (Osaka)	*Moraxella* sp.	—	P
	MR-18	Plant A (Osaka)	*Ochrobactrum anthropi*	98, 12	P, A/C
Soil	YF-1	Forest soil (Yamaguchi)	*S. paucimobilis*	97	P, W, A/C

[a] A mobilizer strain, M1-9, was lost during experiments.
[b] —, not tested.

the introduction of exogenous bacterium with a degradative mobilizable plasmid to enhance the biodegradation capability for a specific contaminant can be a feasible bioaugmentation strategy (i.e. gene bioaugmentation (Pepper et al., 2002)) in some contaminated environments. In particular, it seems possible to

apply gene bioaugmentation to the biological wastewater treatment facility because unintentional dissemination of introduced plasmids into the natural environment can be avoided by disinfection before discharging treated water. Our results also suggest that IncP plasmids may be used as an indicator for judging the implementation of gene bioaugmentation in the target environment.

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References

Birnboim, H. C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*., **7**, 1513–1523.

Cowan, S. T. and Steel, K. J. (1974) Manual for the Identification of Medical Bacteria, 2nd ed., Cambridge University Press, Cambridge.

Davison, J. (1999) Genetic exchange between bacteria in the environment. *Plasmid*, **42**, 73–91.

Disqué-Kochem, C., Battermann, A., Strätz, M., and Dreiseikelmann, B. (2001) Screening for *trbB*- and *traG*-like sequences by PCR for the detection of conjugative plasmids in bacterial soil isolates. *Microbiol. Res*., **156**, 159–168.

Dröge, M., Pühler, A., and Selbitschka, W. (2000) Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Mol. Gen. Genet*., **263**, 471–482.

Götz, A., Pukall, R., Smit, E., Tietze, E., Prager, R., Tschäpe, H., Van Elsas, J. D., and Smalla, K. (1996) Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Appl. Environ. Microbiol*., **62**, 2621–2628.

Joshi, A. K., Baichwal, V., and Ames, G. F. L. (1991) Rapid polymerase chain reaction amplification using intact bacterial cells. *BioTechniques*, **10**, 42–44.

Llanes, C., Gabant, P., Couturier, M., Bayer, L., and Plesiat, P. (1996) Molecular analysis of the replication elements of the broad-host-range RepA/C replicon. *Plasmid*, **26**, 26–35.

Morrissey, J. P., Walsh, U. F., O'Donnnell, A., Moënne-Loccoz, Y., and O'Gara, F. (2002) Exploitation of genetically modified inoculants for industrial ecology applications. *Antonie van Leeuwenhoek*, **81**, 599–606.

Pepper, I. L., Gentry, T. J., Newby, D. T., Roane, T. M., and Josephson, K. L. (2002) The role of cell bioaugmentation and gene bioaugmentation in the remediation of co-contaminated soils. *Environ. Health Perspect*., **110**, 943–946.

Pike, E. B., Carrington, E. G., and Ashburner, P. A. (1972) An evaluation of procedures for enumerating bacteria in activated sludge. *J. Appl. Bacteriol*., **35**, 309–321.

Rochelle, P. A., Fry, J. C., and Day, M. J. (1989) Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed natural suspensions of epilithic bacteria. *J. Gen. Microbiol*., **135**, 409–424.

Soda, S., Watatani, H., Ike, M., and Fujita, M. (1998) Factors affecting the survival of exogenous bacteria in microbial ecosystems: Existence of indigenous bacteria with antagonistic activity. *Biocontrol Sci*., **3**, 63–72.

Top, E., De Smet, I., Verstraete, W., Dijkmans, R., and Mergeay, M. (1994) Exogenous isolation of mobilizing plasmids from polluted soils and sludges. *Appl. Environ. Microbiol*., **60**, 831–839.