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Transfer of Plasmid pJP4 from *Escherichia coli* to Activated Sludge Bacteria by Filter Mating

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Abstract

Conjugation of a self-transmissible, broad-host-range, mercury resistant plasmid pJP4 harbored in *Escherichia coli* HB101 into activated sludge bacteria was investigated. Filter mating experiments were performed on the nutrient agar plate with *E. coli* HB101 (pJP4) and the activated sludge bacteria as the donor and the recipients for conjugation, respectively. Transfer frequencies were 2.0×10^{-1} – 5.8×10^{-1} per donor and 2.2×10^{-1} per potential recipient. Most of the transconjugants were inferentially identified as *Burkholderia cepacia* and *Sphingomonas paucimobilis*, which are common bacteria in the activated sludge process. Some transconjugants showed higher resistance to $HgCl_2$ (up to 100 mg/*l*) than the original host of pJP4, *Cupriavidus necator* JMP134 (50 mg/*l*).

Key words: activated sludge; conjugation; indigenous transconjugant; plasmid

INTRODUCTION

Genes related to metal resistance or contaminant degradation are often located on plasmids. Bacterial inoculants used for bioaugmentation in wastewater treatment often contain such plasmids[1,2]. However, the introduced population is often unable to adapt and rapidly declines in microbial ecosystems such as activated sludge by biotic and abiotic factors[1,3]. Those experiences suggest that plasmid transfer from introduced bacteria to indigenous predominant bacteria in activated sludge will enhance the bioaugmentation possibility by providing ecologically stable hosts for the plasmid: so-called plasmid-mediated augmentation[2,4]. Furthermore, indigenous transconjugants might highly express metal resistance or contaminant degradation abilities than the introduced donor strain by rearrangement or combination of the plasmid genes with pre-existing genes[2]. However, a few studies evaluated the potential for plasmid transfer from introduced donors to indigenous recipients in activated sludge. Our previous report elucidated that the actual host range of an antibiotic resistant plasmid RP4[5] depends on the existing bacterial consortium in the activated sludge process[6].

In this study, pJP4–a self-transmissible and mercury resistant plasmid[7,8]–was used as a model plasmid for plasmid-mediated bioaugmentation. Although numerous reports have described the transfer of pJP4 in soil microcosms[9-13], only Bathe *et al.*[14] reported *in situ* transfer of pJP4 in an activated sludge sample. To obtain information related to the host range of plasmid pJP4 in activated sludge, conjugation experiments were performed under conditions that were most likely to lead to high transfer rates: with high cell density on a nutrient agar surface, but with a complex activated sludge derived

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recipient community. Indigenous transconjugants harboring pJP4 were isolated from activated sludge and characterized by their phenotypic mercury resistance.

MATERIALS AND METHODS

Bacterial strains *Cupriavidus necator* JMP134 (pJP4) (formerly *Ralstonia eutropha*) was obtained from DSMZ, Germany. This 88-kb broad-host-range plasmid of incompatibility group P–1β harbors genes related to mercury resistance and key catabolic functions for 2.4–dichlorophenoxyacetic acid (2,4–D)[7,8]. The *E. coli* K–12 strain HB101 is a restriction-negative streptomycin (Sm)–resistant strain that requires thiamine, leucine, and proline for growth[15]. Activated sludge samples were collected in autumn 2004 from wastewater treatment plants (WWTPs) M and S in Osaka prefecture: M–WWTP uses a step aeration process and treats mainly domestic wastewater at 50,000 m^3/d, whereas S–WWTP uses an anoxic–oxic process and treats mainly domestic wastewater at 20,000 m^3/d. Mixed-liquor suspended solids of both plants samples were about 1,500 mg/*l*. Heterotrophic bacteria and enteric bacteria were counted respectively on R2A agar plates (Merck and Co. Inc., Darmstadt, Germany) and deoxycholate agar plates (Eiken Chemical Co. Ltd., Tokyo). Transconjugants were identified using the api20E or the api20NE system (API System S.A., Montalieu-Vercieu, France).

Filter mating As a preliminary experiment, conjugation experiment of pJP4 was conducted from *C. necator* JMP134 to *E. coli* HB101. The donor strain *C. necator* JMP134 (pJP4) grown to the mid-log phase in 2.5 m*l* of a modified LB medium (bacto peptone 10 g/*l*, yeast extract 5.0 g/*l*, NaCl 5.0 g/*l*, pH 7.2) with $HgCl_2$ 10 mg/*l* was harvested by centrifugation (10,000 × *g*, 10 min, 4℃) and washed twice with 5 mg/*l* sodium tripolyphosphate (TPP). The recipient strain *E. coli* HB101, after it had grown to the mid-log phase in 2.5 m*l* of the modified LB medium with Sm 50 mg/*l*, was harvested by centrifugation and washed twice with 5 mg/*l* TPP. The mixture of the donor cells and the recipient cells was filtered through a 0.45–μm cellulose ester filter (A045B047; Advantec, Ehime). The filter was transferred onto R2A agar plates. After incubation for 24 h at 28℃, the filter was transferred to a test tube and vortexed for 5 min in 10 m*l* of TPP to detach bacteria. Serial dilutions were then spread onto agar plates and incubated. Then *C. necator* JMP134 (pJP4) was enumerated using the modified LB agar plate with $HgCl_2$ 10 mg/*l*, *E. coli* HB101 using deoxycholate agar with Sm 50 mg/*l*, transconjugant *E. coli* HB101 (pJP4) using desoxycholate agar with $HgCl_2$ 10 mg/*l* and Sm 50 mg/*l*. The plates were incubated for 2 days at 28℃.

Activated sludge samples (5 m*l* with 45 m*l* of TPP) were treated using a sonicator (UD201; Tomy Seiko Co. Ltd., Tokyo) to disperse the bacterial cells from the sludge flocs. The donor strain *E. coli* HB101 (pJP4), after it had grown to the mid-log phase in 2.5 m*l* of the modified LB medium with $HgCl_2$ 10 mg/*l* and Sm 50 mg/*l*, was harvested by centrifugation, washed twice with 5 mg/*l* TPP, and suspended in 5.0 m*l* of the activated sludge sample. The mixture was applied for filter mating in the same manner as that used for the preliminary experiment. Potential recipients were enumerated using a basal salt medium (BSM)[6] containing glucose 2.0 g/*l* as the sole carbon source, and transconjugants using BSM containing glucose 2.0 g/*l* with $HgCl_2$ 10 mg/*l*. In addition, cycloheximide 50 mg/*l* was added to all solid media to prevent fungal growth on the plates. The plates were incubated for 4–7 days at 28℃. This detection technique for transconjugants was based on the donor's nutrient requirements. Only glucose-using bacteria without requirements for the amino acids, and with resistance to $HgCl_2$ can grow on the medium for transconjugants. Control experiments were conducted using the activated sludge sample without addition of the donor strain.

Plasmid isolation and gel electrophoresis Randomly chosen colonies were isolated from the transconjugant-selective plates to confirm the presence of pJP4 in transconjugants. Transconjugant strains were grown in the modified LB medium with $HgCl_2$ 10 mg/*l* overnight in a reciprocal shaker (120 rpm) at 28℃. Then they were used for plasmid extraction. The extraction of plasmid DNA

was performed using a slightly modified Birnboim and Doly method[16]. The crude plasmid DNA samples were analyzed using electrophoresis in 0.6% (w/v) agarose gels in Tris-acetate-EDTA buffer. The gel was stained using ethidium bromide of 0.5 μl/ml; it was then visualized under UV light.

Detection of *tfdB* gene on pJP4 Transconjugant strains were grown on modified LB plates with $HgCl_2$ at 28℃ for 2–4 days. Then they were used for PCR-based detection of a specific gene on pJP4. Oligonucleotide primers 591 and 592 were used to amplify a region of *tfdB* gene on the plasmid[17]. Subsequent PCR amplification of DNA from individually plucked colonies was conducted in a 20-μl reaction mix containing *Taq* DNA polymerase and the primer pair for 25 cycles with denaturation at 94℃ for 90 s and extension at 72℃ for 60 s, and final extension at 72℃ for 7 min using a thermal cycler. Aliquots of 5.0 μl of the PCR products (205 bp) were analyzed using 1.5% (w/v) agarose gel stained with ethidium bromide.

Mercury resistance of transconjugants The degree of mercury resistance of transconjugants was assayed according to the colony forming ability on nutrient agar plates containing varying concentrations of $HgCl_2$. Bacterial colonies grown on the selective plates were transferred to the new agar plates. Glucose-containing BSM agar plate was used for transconjugants isolated activated sludge samples. The modified LB agar plate was used for *C. necator* JMP134 (pJP4). Deoxycholate agar plate was used for *E. coli* HB101 and *E. coli* HB101 (pJP4). The plates were incubated for 7 days at 28℃.

RESULTS

Transfer frequency of pJP4 from *C. necator* to *E. coli* As a preliminary experiment, pJP4 was transferred from the original donor to the laboratory *E. coli* strain. The transfer frequency of pJP4 from *C. necator* JMP134 to *E. coli* HB101 is shown in Table 1. The *tfdB* gene was detected from all selected transconjugant colonies using PCR. Plasmid DNA preparation performed with the transconjugant showed the presence of a plasmid with a similar size to pJP4. The code number of the api20E system for the obtained transconjugant was 504452, implying that this transconjugant belongs to *E. coli*. These results indicate that *E. coli* HB101 (pJP4) was obtained in the filter mating experiment.

Transfer frequency of pJP4 from *E. coli* to activated sludge bacteria Colony counts of heterotrophic bacteria in the activated sludge samples are presented in Table 2. Enteric bacteria counted on deoxycholate agar plates were only about 0.1% of the heterotrophic bacteria enumerated on R2A agar plates. Colony counts of the potential recipients on glucose-containing BSM agar plates were 2.7–4.5% of the heterotrophic bacteria.

In situ transfer experiments were conducted for each activated sludge sample from M-WWTP and S-WWTP. The transfer frequency of pJP4 from the donor *E. coli* HB101 to the indigenous activated sludge bacteria is presented in Table 3. There were 2.6×10^7 – 3.3×10^7 CFU/filter transconjugants (transconjugant-characteristic phenotypes) after 24-h-filter mating. Transfer frequencies

Table 1 Transfer of plasmid RP4 from *C. necator* JMP134 to *E. coli* HB101 by filter mating

Colony counts (CFU/filter)			Transfer frequency (–)	
Donor (D)	Recipient (R)	Transconjugant (T)	T/D	T/R
2.4×10^7	2.0×10^8	2.2×10^4	9.1×10^{-4}	1.1×10^{-4}

Table 2 Colony counts of bacteria in activated sludge sampled from WWTPs (CFU/ml)

	M-WWTP	S-WWTP
R2A agar	1.7×10^7	5.3×10^7
Deoxycholate agar	3.0×10^4	5.0×10^4
Glucose-containing BSM agar	4.7×10^5	2.4×10^6

were 2.0×10^{-1} – 5.8×10^{-1} per donor and 2.2×10^{-1} per potential recipient. The background levels of the transconjugant-characteristic phenotypes were less than 10^5 CFU/filter in the activated sludge samples without addition of the donor strain.

Verification of presence of pJP4 in transconjugants Three and four transconjugants with the stable transconjugant-phenotype were isolated, respectively, from indigenous activated sludge bacteria from M–WWTP and S–WWTP, as shown in Table 4. The *tfdB* gene was detected from all of the seven selected transconjugant colonies using PCR. On the other hand, the *tfdB* gene was not detected from the transconjugant-characteristic phenotypes isolated from the activated sludge samples without addition of the donor strain. In addition, plasmid DNA preparation performed with the seven transconjugants showed the presence of a plasmid with similar size to that of pJP4 (88 kb). These results indicate that the transconjugant-characteristic phenotypes detected in the filter mating experiment actually possessed plasmid pJP4.

In addition, plasmid DNA with different sizes to pJP4 existed in a transconjugant, bacterial strain S13, suggesting the presence of naturally occurring plasmids in indigenous activated sludge bacteria. Although the sizes of these plasmids have not been determined exactly, the bacterial strain S13 had a slightly larger plasmid than pJP4.

Characterization of transconjugants Characteristics of the transconjugants are presented in Table 4. All transconjugants except bacterial strain S6 from S–WWTP were inferred as typical predominant activated sludge bacteria, *Burkholderia cepacia* and *Sphingomonas paucimobilis*. Tolerable mercury concentrations of the transconjugants are listed in Table 5. The original host of pJP4, *C. necator* JMP134, grew on the agar plate with $HgCl_2$ concentrations as high as 50 mg/*l*. Although *E. coli* HB101 was unable to grow on the plate with $HgCl_2$ even at 10 mg/*l*, pJP4 gave *E. coli* HB101 mercury resistance up to 100 mg/*l*. The activated sludge-derived transconjugants showed various tolerable concentrations to mercury. The strain M26 (pJP4) showed lower mercury resistance (10 mg/*l*) but strains S6 (pJP4) and M46 (pJP4) had higher mercury resistance up to 100 mg/*l* than *C. necator* JMP134 (pJP4).

Table 3 Transfer of plasmid pJP4 from *E. coli* HB101 to activated sludge bacteria by filter mating

Activated sludge sample	Colony counts (CFU/filter)			Transfer frequency	
	Donor (D)	Potential recipient (R)	Transconjugant (T)	T/D	T/R
M–WWTP	4.4×10^7	1.1×10^8	2.6×10^7	5.8×10^{-1}	2.2×10^{-1}
S–WWTP	1.6×10^8	1.5×10^8	3.3×10^7	2.0×10^{-1}	2.2×10^{-1}

Table 4 Inferential identification of transconjugant activated sludge bacteria harboring plasmid pJP4

Isolate	Species	Gram stain	Oxidase	Catalase	OF test	Api20NE No.
M–WWTP						
Strain M26	*Burkholderia cepacia*	–	+	+	–	1047775
Strain M33	*Sphingomonas paucimobilis*	–	+	+	O	0067740
Strain M46	*Burkholderia cepacia*	–	+	+	O	0067757
S–WWTP						
Strain S2	*Burkholderia cepacia*	–	+	+	O	1067477
Strain S4	*Burkholderia cepacia*	–	+	+	O	1067577
Strain S6	Unidentified	–	–	+	–	0200000
Strain S13	*Sphingomonas paucimobilis*	–	+	+	O	1463740

Table 5 Mercury resistance of transconjugant–activated sludge bacteria

Bacterial strain	$HgCl_2$ concentration (mg/*l*)			
	10	25	50	100
C. necator JMP134(pJP4)	+	+	+	–
E. coli HB101	–	–	–	–
E. coli HB101(pJP4)	+	+	+	+
Strain M26(pJP4)	+	–	–	–
Strain M33(pJP4)	+	+	+	–
Strain M46(pJP4)	+	+	+	+
Strain S2(pJP4)	+	+	+	–
Strain S4(pJP4)	+	+	+	–
Strain S6(pJP4)	+	+	+	+
Strain S13(pJP4)	+	+	–	–

+, growth; –, no growth on each plate.

DISCUSSION

Diversity of detectable transconjugants depends on various mating conditions and isolation strategies[17]. In this study, the conjugation experiments were performed at a high cell density on a nutrient agar surface. Filter mating frequencies are generally at least 10–fold greater than plate mating and broth mating frequencies[18]. In our previous report[6], the transfer frequencies of plasmid RP4 from *E. coli* to activated sludge bacteria (5.1×10^{-2} – 7.5×10^{-1} per donor and 4.6×10^{-3}–7.0×10^{-2} per potential recipient) were as high as those of pJP4 in this study (Table 3). In this technique, detectable bacteria depend on the carbon source, but glucose is an easily utilizable carbon sources for most bacteria. We might have underestimated the range of potential recipients and transconjugants because bacterial strains that could not grow on the R2A medium and the glucose-containing BSM nor express mercury resistance have been missed using this approach. Although our method also is affected by those limitations, it was demonstrated to be useful for a feasibility test of plasmid-mediated augmentation in the activated sludge process.

Transconjugant strains belonging to *B. cepacia* and *S. paucimobilis* were isolated in this study using *E. coli* as a donor and activated sludge bacteria as recipients in two WWTPs (Table 4). Species of *Burkholderia*, *Ralstonia*, and *Pseudomonas* have been found most frequently as pJP4 hosts in soil microcosms[9–12]. The only related report in the relevant literature is that of Bathe *et al.*[14], who described the transfer of pJP4 from *Pseudomonas putida* to an activated sludge bacteria sampled from a WWTP in Germany. In their study, the transconjugants isolated by flow cytometry belonged to various genera of the α–, β–, γβ– and γ–classes of the *Proteobacteria*, mostly to the families *Rhizobiaceae* and *Comamonadaceae* and the genus *Stenotrophomonas*[14]. Because the number of the transconjugants isolated in this study was limited, further studies on the host range of pJP4 in activated sludge are needed.

In our previous report[6], the transfer of plasmid RP4 from *E. coli* to activated sludge bacteria sampled from M–WWTP and S–WWTP was investigated. Of the 15 transconjugants from the WWTPs, 4 were typical activated sludge bacteria (*Ochrobactrum anthropi*, *P. fluorescens*, and *P. putida*); the remaining 12 were enteric bacteria (*Citrobacter freundii*, *Enterobacter cloacae*, *E. coli*, and *Klebsiella pneumoniae* spp. *pneumoniae*) which were the minor population in activated sludge (Table 2). Those results indicated that IncP–1β plasmid pJP4 and IncP–1α plasmid RP4 transfer to quite different bacteria in activated sludge, even in a similar combination with *E. coli* as the donor and the activated sludge bacteria as recipients.

As we expected, some transconjugants obtained from activated sludge samples showed higher mercury resistance than *C. necator* JMP134 (Table 5). Mercury is detoxified by bacterial reduction of Hg^{2+} to Hg^0 and diffusional loss of Hg^0 from the cell. The cellular machinery encoded by the microbial *mer* operon on pJP4 provides specific uptake proteins (MerP and MerT) which transport Hg^{2+} in the cytoplasm of the host cell and which prevent damage to the cell. In the cell, Hg^{2+} is reduced with NADPH to Hg^0 by the enzyme mercuric reductase (MerA), which is related to glutathione reductase[8]. The rate-limiting step for the overall detoxification reaction is probably uptake into the host cell, rather than

reduction of Hg^{2+} in the cell[19]. Although rearrangement of the plasmid genes with pre-existing genes were not investigated, the cell-surface characteristics of the trans-conjugants can at least partially explain the different degrees of the phenotypic mercury resistance on the agar plates.

In conclusion, the self-transmissible and mercury resistant plasmid pJP4 can be disseminated to dominant bacterial species in activated sludge. It can therefore express its capability in activated sludge processes. Additional studies of long-term survival of the plasmid and the transconjugants are necessary for verification of plasmid-mediated augmentation in activated sludge processes.

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