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Effects of Genetically Different 2,4-D-degradative Plasmids on Degradation Phenotype and Competitiveness of Soil Microorganisms

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The effects of various 2,4-D-degradative plasmids on the axenic growth patterns, the degradation phenotypes, and the competitiveness of different host bacteria were evaluated in liquid cultures; the organisms and plasmids used were *Alcaligenes eutrophus* JMP134/pJP4, *Alcaligenes paradoxus*/p2811, *Pseudomonas pickettii*/p712, *Pseudomonas pseudomallei*/p745, *Pseudomonas cepacia*, and *Alcaligenes* JMP228. The 2,4-D-degradative plasmids p745, pJP4, and p712 or p2811 exhibited very different restriction fragment profiles in restriction endonuclease digests. These plasmids were transferred to the recipients (*P. cepacia* and *Alcaligenes* JMP228) at relatively high frequencies ranging from 8.9×10^{-3} to 1.6×10^{-5} per donor cell. In the axenic liquid cultures the fast-growing strains, such as *P. pseudomallei*/p745 and *P. cepacia*/pJP4, exhibited short lag periods, high specific growth rates, and high relative fitness coefficients, while the slow-growing strains, such as *P. pickettii*/p712 and *A. paradoxus*/p2811, had long lag periods, low specific growth rates, and low relative fitness coefficients. Depending on the type of plasmid containing the genes for the 2,4-D pathway, some transconjugants exhibited intermediate growth patterns between the fast-growing strains and the slow-growing strains. The plasmid and plasmid-host interactions determined specific growth rate and lag time, respectively, which were shown to be principal determinants of competitiveness among the strains, but relative fitness coefficient derived from the axenic culture was not always predictive for the mixed culture condition.

Key words: 2,4-D-degradative plasmid, competitiveness, specific growth rate, lag time, relative fitness coefficient

The use of naturally occurring or genetically-engineered microorganisms (GEMs) for the detoxification of contaminated environment is getting more attention because of their potential for effectiveness and cost-competitiveness (14, 18). The indigenous microbial population may be stimulated to degrade the toxic pollutants by optimizing nutrient and environmental conditions to enhance their activities (10, 15). It is also possible that microorganisms are genetically engineered in the laboratory and inoculated into the contaminated environment to efficiently remove the chemical pollutants (1, 3, 12, 13). This approach will be particularly valuable for sites where the appropriate indigenous microorganisms are not present.

Most of the pesticide-degradative genes are known to be carried on conjugative plasmids (2, 4, 18). This may make it easy to develop new microorganisms with enhanced degradation capability, since the degradative genes can easily be isolated, identified, manipulated, and transferred to new host bacteria through microbial conjugation and electroporation.

In the previous study, a variety of pesticide-degrading bacteria were isolated from agricultural soils and it was shown that not only the bacterial isolates were highly

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diverse in their physiological and taxonomic properties, but also their pesticide-degrading plasmids were genetically diverse (8). It was also demonstrated that the complex interaction between the plasmid and the host chromosomal DNA could lead to the genetic rearrangement of pesticide-degradative genes under a specific selection condition, resulting in more efficient utilization of the pesticide (7). Since the host background is directly related to the physiological and phenotypic properties of GEMs and plays an important role in their persistence in the open environment, it will be desirable to investigate the role of the host background versus the role of plasmid in determining the growth patterns and competitiveness of pesticide-degrading bacteria.

In this study we examined the transfer of various 2,4-D-degradative plasmids to different host bacteria, and the effects of different 2,4-D-degradative plasmids on both the growth patterns and the degradation phenotypes of the host bacteria were evaluated. In addition, batch liquid cultures were used to study the competitiveness of the original strains and the transconjugants in the mixed cultures.

Materials and Methods

Bacterial strains

Strains of *Pseudomonas pseudomallei*/p745, *Alcaligenes eutrophus* JMP134/pJP4, *Pseudomonas pickettii*/p712, and *Alcaligenes paradoxus*/p2811, each of which had a respective 2,4-D-degradative plasmid, were used as donors in matings. In the previous study, plasmids p745 and pJP4 were grouped into hybridization group I and plasmids p712 and p2811 were grouped into hybridization group II (8). *Pseudomonas cepacia* and *Alcaligenes* JMP228, which were 2,4-D$^-$ (2,4-dichlorophenoxyacetic acid), 3-CB$^-$ (3-chlorobenzoic acid), and MCPA$^-$ (2-methyl-4-chlorophenoxyacetic acid), were used as recipients. *P. cepacia* contains transposon Tn5 and is resistant to kanamycin (75 μg/ml), bacitracin (50 μg/ml), and carbenicillin (50 μg/ml). *Alcaligenes* JMP228 is resistant to rifampicin (100 μg/ml) through spontaneous mutation.

Media and growth conditions

Peptone-tryptone-yeast extract-glucose medium (7) was used for strain purification and enumeration. MMO mineral medium (19) plus 2,4-D at 500 ppm was used for the axenic culture and competition experiment.

DNA isolation and detection

Plasmid DNA was isolated by using the procedure of Hirsch *et al.* (6). To analyze the restriction fragment profiles, purified plasmid DNA was digested with *Eco*RI restriction endonuclease and separated in a horizontal 0.7% agarose gel (17). For detection of plasmid DNA, cells were lysed by using a modified form (7) of the procedure of Kado and Liu (11).

Conjugation

Matings were performed on membrane filters as described by Willetts (20). Transconjugants were selected on 2,4-D minimum medium containing MMO mineral medium, 2,4-D at 500 ppm, and the appropriate antibiotics. The frequency of transfer was calculated as the number of exconjugants per donor cell.

Degradation analysis

Each strain was cultured in MMO mineral medium with 2,4-D (500 ppm) or sodium acetate (250 ppm) as the sole carbon source to produce cells induced or uninduced to metabolize 2,4-D. Cultures were grown at 30℃ and aerated by shaking at 200 rpm in an incubator shaker. Cells in the late log phase were harvested by centrifugation at 10,000 × g for 10 min at 4℃, washed twice with an equal volume of 15 mM phosphate buffer (pH 7.0), and resuspended in the same buffer. Aliquots of suspended cells were inoculated into culture tubes, each of which contained MMO mineral medium supplemented with 2,4-D, 3-CB, or MCPA at a concentration of 250 ppm. The cultures were shaken at 30℃ for 2 weeks, after which the optical density at 550 nm was determined. To determine the degradation of phenoxyacetates, the cultures were centrifuged to remove the cellular material, and the UV absorption was measured at 283 nm, 284 nm, and 279 nm for 2,4-D, 3-CB, and MCPA, respectively.

Competition experiments

Bacterial strains which were cultured, harvested, and prepared in 15 mM sodium phosphate buffer (9) were used for axenic and mixed culture experiments. The mixed culture studies were performed with two or three strains under induced condition, each inoculated at the similar density ($\sim 1.0 \times 10^6$ cells/ml) in the 2,4-D mineral medium (500 ppm). Whenever the mixed culture reached the maximum growth, an aliquot of culture (0.1%) was repeatedly transferred into fresh 2,4-D medium up to 5 times, and individual strains were distinguished and counted on the basis of distinctive colony morphologies. Liquid culture studies were performed in duplicate.

Results and Discussion

Restriction fragment profiles

Analysis with restriction endonuclease *Eco*RI revealed

Table 1. Restriction patterns of 2,4-D-degradative plasmids.

Plasmid	DNA fragment size with *Eco*RI digestion (kb)	Size of plasmid (kb)
p712	18.5, 6.7, 5.7, 3.9, 3.5, 1.7, 0.8	40.8
p745	16.5, 8.2, 7.7, 7.1, 6.5, 5.3, 4.8, 2.4, 2.1, 2.0, 1.9, 1.7, 1.1, 0.9, 0.5	68.7
p2811	18.5, 8.2, 6.1, 3.9, 3.5, 1.7, 0.8	42.7
pJP4	28.6, 16.3, 11.2, 9.4, 7.9, 3.2, 2.3, 1.7, 1.3	81.9

Table 2. Transfer frequency[a] of 2,4-D-degradative plasmids.

Donor	Recipient	
	Alcaligenes spp.	*P. cepacia*
Alcaligenes JMP134/pJP4	1.3×10^{-3}	1.6×10^{-5}
A. paradoxus/p2811	1.5×10^{-3}	6.2×10^{-3}
P. pickettii/p712	1.6×10^{-5}	8.9×10^{-3}
P. pseudomallei/p745	4.1×10^{-5}	1.1×10^{-3}

[a] Values are means of two independent matings.

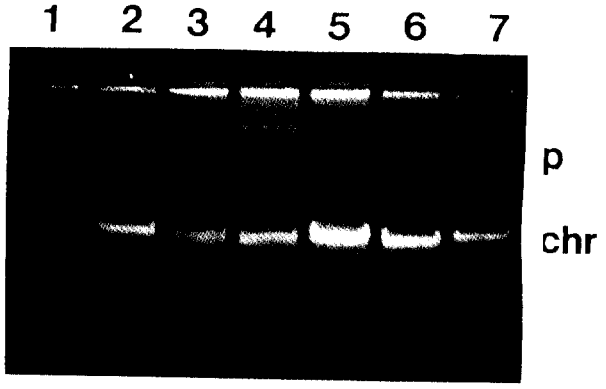

Fig. 1. Agarose gel showing plasmids in the donors and transconjugants. Lanes: 1, recipient *P. cepacia*; 2, transconjugant *Alcaligenes* JMP228/p745; 3, transconjugant *Alcaligenes* JMP228/pJP4; 4, transconjugant *P. cepacia*/pJP4; 5, recipient *Alcaligenes* JMP228; 6, transconjugant *Alcaligenes* JMP228/p2811; 7, transconjugant *Alcaligenes* JMP228/p712.

different banding patterns among p745, pJP4, and p712 or p2811, but similar banding patterns were obtained between p712 and p2811 (Table 1). For p712 and p2811, only two of the seven fragments showed altered mobilities in agarose gel electrophoresis, indicating that these two plasmids are physically similar to each other.

Plasmid transfer

To study the phenotypic and competitive characteristics of the isolated 2,4-D-degradative plasmids in the new host background, all of the plasmids were transferred by filter mating from their original hosts to the recipients, *P. cepacia* and *Alcaligenes* JMP228. The 2,4-D-degradative plasmids were shown to be transferred at relatively high frequencies ranging from 8.9×10^{-3} to 1.6×10^{-5} (Table 2). The transfer frequencies were generally higher in matings between the same genera than between the different genera. The respective plasmid bands were observed in agarose gels containing the transconjugants (Fig. 1).

Table 3. Growth characteristics of 2,4-D degrading bacteria in axenic culture[a].

Strain	Lag period (h)	Specific growth rate (h^{-1})	Relative fitness coefficient[b]
P. pseudomallei/745	<15	0.132	1.0
Alcaligenes JMP228/p745	<15	0.202	0.99
P. cepacia/p745	<15	0.133	0.83
A. eutrophus/pJP4	25~40	0.162	~0
Alcaligenes JMP228/pJP4	<15	0.187	0.89
P. cepacia/pJP4	<15	0.148	0.98
P. pickettii/p712	>50	0.071	~0
Alcaligenes JMP228/p712	25~40	0.047	0.31
P. cepacia/p712	25~40	0.052	0.34
A. paradoxus/p2811	>50	0.043	~0
Alcaligenes JMP228/p2811	25~40	0.049	0.15
P. cepacia/p2811	25~40	0.048	0.17

[a] All values represent means from two independent liquid cultures. [b] The relative fitness coefficient was determined in axenic broth culture according to reference (16), i.e., ratio of the number of doublings of each strain to that of *P. pseudomallei* under the same conditions. The first 30 h of incubation was chosen to evaluate relative fitness since the most rapidly growing strain, *P. pseudomallei*, stopped growing at this time due to depletion of substrate.

Axenic culture

To evaluate the effect of the host cell background versus the effect of the 2,4-D-degradative plasmid on the growth patterns of 2,4-D degraders, the original strains and transconjugants were grown in 2,4-D mineral medium under uninduced condition, and the lag period, specific growth rate (5), and relative fitness coefficient (16) were determined (Table 3). *P. pseudomallei*/p745, *Alcaligenes* JMP228/p745, *P. cepacia*/p745, *Alcaligenes* JMP228/pJP4, and *P. cepacia*/pJP4 exhibited short lag periods (<15 h) and began to grow exponentially after about 20 h of incubation (Fig. 2). The specific growth rates of these bacteria ranged from 0.132 h^{-1} to 0.202 h^{-1}, with *Alcaligenes* JMP228/p745 showing the highest value. *A. eutrophus* JMP134/pJP4, *Alcaligenes* JMP228/p712, *P. cepacia*/p712, *Alcaligenes* JMP228/p2811, and *P. cepacia*/p2811 exhibited a longer lag periods (25~40 h), and *P. pickettii*/p712 and *A. paradoxus*/p2811 exhibited the longest lag periods (>50 h). Overall, plasmids p745 and pJP4 allowed the host bacteria to grow more rapidly

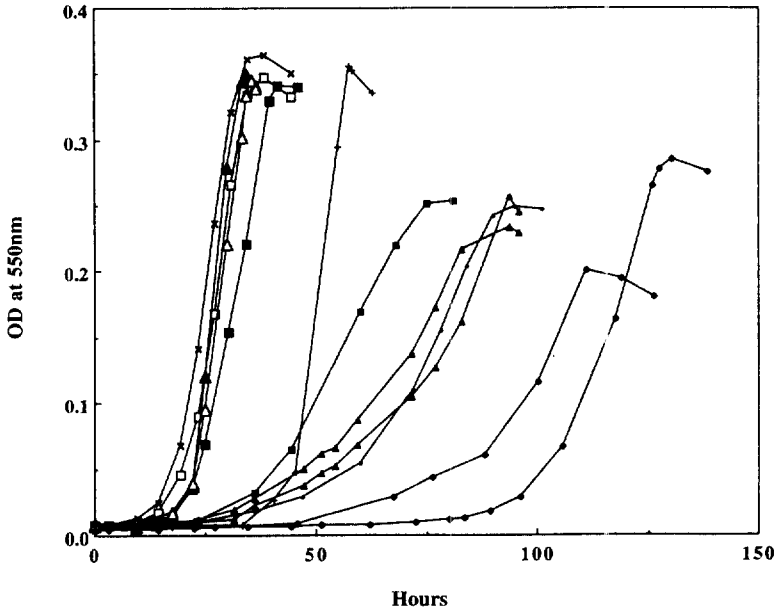

Fig. 2. Growth patterns of 2,4-D-degrading bacteria in axenic culture. Symbols: x, *P. pseudomallei*/p745; ▲, *Alcaligenes* JMP228/p745; △, *Alcaligenes* JMP228/pJP4; □, *P. cepacia*/pJP4; ■, *P. cepacia*/p745; +, *A. eutrophus* JMP134/pJP4; ▪, *P. cepacia*/p712; ▲, *Alcaligenes* JMP228/p712; ○, *P. cepacia*/p2811; △, *Alcaligenes* JMP228/p2811; ◇, *A. paradoxus*/p2811; ◆, *P. pickettii*/p712. Each point is the mean for two replicate liquid cultures. OD, optical density.

than plasmids p712 and p2811. With the plasmids pJP4, p712, and p2811, the new host *Alcaligenes* JMP228 and *P. cepacia* exhibited shorter lag periods and higher relative fitness coefficients than the original hosts. *P. pseudomallei*/p745 had the largest number of doublings during the first 30 h of incubation and had the highest fitness coefficient among these 2,4-D degraders, suggesting that it might be the best competitor.

Degradation analysis

Each transconjugant was grown on medium containing 2,4-D or acetate as the sole carbon source and then examined for any change in their ability to degrade other compounds related to 2,4-D (Table 4). The plasmid pJP4, which is known to have degradative genes for 2,4-D, 3-CB, and MCPA, allowed the new host bacteria to utilize these compounds as the sole carbon sources. The new host *Alcaligenes* JMP228, which is MCPA$^-$, utilized MCPA as the sole carbon source with any of the plasmids used. This result suggests that this strain may have part of the MCPA-degradative pathway in its chromosome, thus allowing complete degradation of MCPA with plasmids p712, p2811, or p745. One interesting observation was that the plasmid p745 exhibited different phenotype in different host background. While the original host *P. pseudomallei* slightly grew on 3-CB, the new host *Alcaligenes* JMP228 vigorously grew on 3-CB in the presence of p745. Moreover, the other new host *P. cepacia*,

Table 4. Patterns of utilization[a] of herbicides by transconjugants.

Strain	Growth condition	Substrate		
		2,4-D	3-CB	MCPA
A. eutrophus JMP134/pJP4	A	++	++	++
	U	++	++	++
Alcaligenes JMP228/pJP4	A	++	++	++
	U	++	++	++
P. cepacia/pJP4	A	++	++	++
	U	++	++	++
P. pickettii/p712	A	++	−	−
	U	++	−	+
Alcaligenes JMP228/p712	A	++	−	++
	U	++	−	++
P. cepacia/p712	A	++	−	−
	U	++	−	−
A. paradoxus/p2811	A	++	−	−
	U	++	−	−
Alcaligenes JMP228/p2811	A	++	−	++
	U	++	−	++
P. cepacia/p2811	A	++	−	−
	U	++	−	−
P. pseudomallei/p745	A	++	+	−
	U	++	+	−
Alcaligenes JMP228/p745	A	++	++	++
	U	++	++	++
P. cepacia/p745	A	++	++	−
	U	++	−	−

[a] The bacteria were grown on 2,4-D (A) or on acetate (U) and then tested for substrate utilization capabilities. ++, >80% reduction in peak height from UV scanning and substantial growth (OD_{550}>0.13); +, 40 to 60% reduction in peak and moderate growth (OD_{550}>0.08); −, <15% reduction in peak and very scant growth (OD_{550}<0.01).

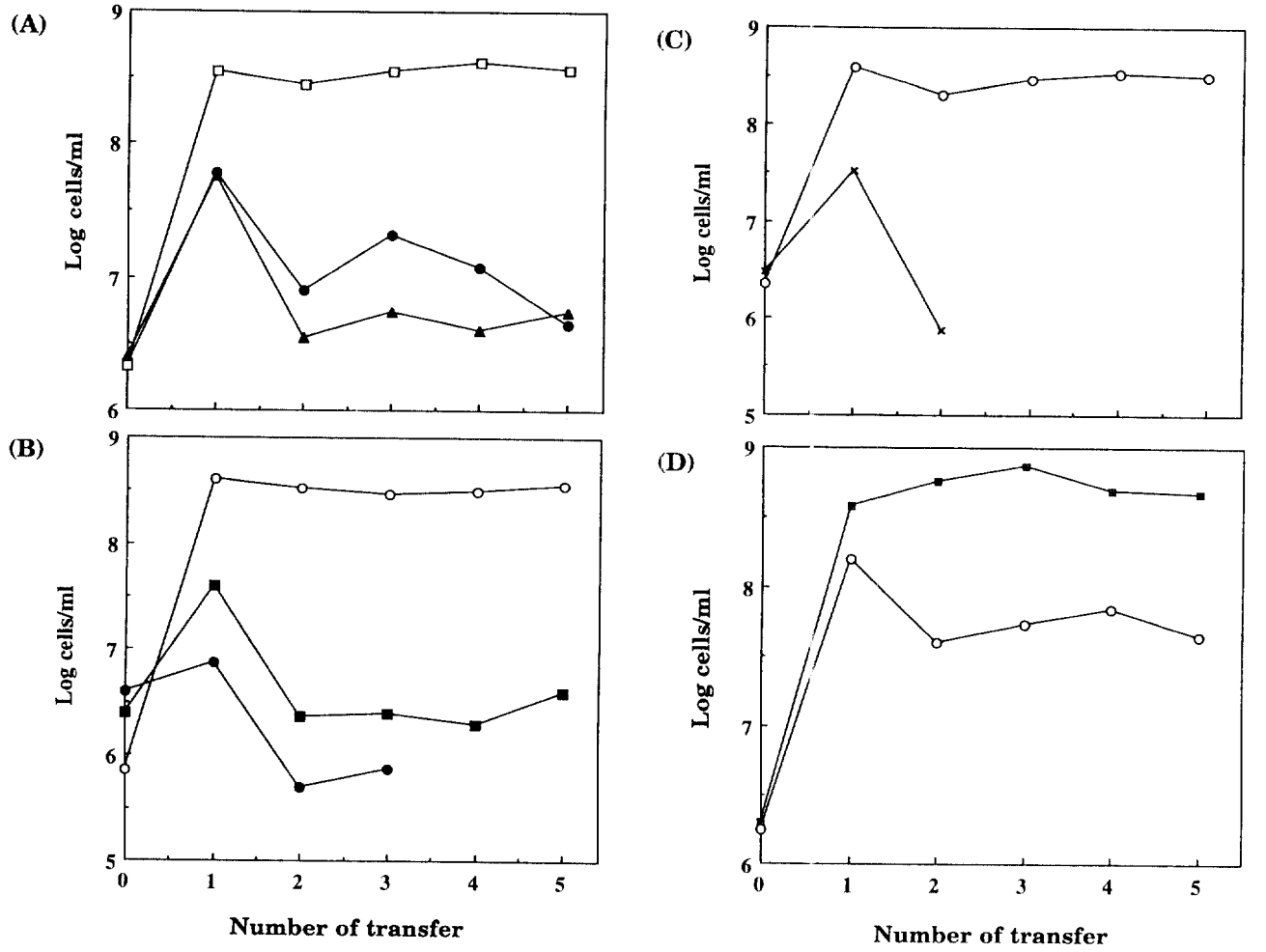

Fig. 3. (A) Competition for 2,4-D among *Alcaligenes* JMP228/p2811 (□), *P. cepacia*/p2811 (▲), and *A. paradoxus*/p2811 (●). (B) Competition for 2,4-D among *P. cepacia*/pJP4 (○), *P. pickettii*p712 (■), and *A. paradoxus*/p2811 (●). *A. paradoxus*/p2811 was not detected after the 3rd transfer. (C) Competition for 2,4-D between *P. cepacia*/pJP4 (○) and *P. pseudomallei*/p745 (×). *P. pseudomallei*/p745 was not detected after the 2nd transfer. (D) Competition for 2,4-D between *Alcaligenes* JMP228/p745 (□) and *P. cepacia*/pJP4 (○). The data are the means for two replicate liquid cultures.

which is 3-CB$^-$, could degrade 3-CB with p745 under induced condition, suggesting that this compound was probably metabolized in this new host background because of its structural similarity to 2,4-D.

Plasmid stability

To analyze the stability of the 2,4-D-degradative plasmid in the new host, each transconjugant was grown in LB medium up to about 100 generations and then 30 colonies were inoculated into 2,4-D mineral medium to examine for their ability to degrade 2,4-D. All of the plasmids used seemed to be stably maintained in the original host bacteria, because all the colonies tested degraded 2,4-D. However, one out of each 30 colonies of transconjugants *P. cepacia*/p712 and *P. cepacia*/p745 failed to degrade 2,4-D, indicating that these plasmids probably were less stably maintained in this new host.

Competition experiment

The competitiveness of the transconjugant was monitored by plate counting in two or three-member liquid cultures under induced condition. When *Alcaligenes* JMP 228/p2811, *P. cepacia*/p2811, and *A. paradoxus*/p2811 were inoculated together into 2,4-D mineral medium to evaluate the competitiveness among different strains containing the same plasmid, *Alcaligenes* JMP228/p2811 was observed to outgrow the other two strains (Fig. 3-A). This result suggested that host *Alcaligenes* JMP228 could be a better competitor than hosts *P. cepacia* and *A. paradoxus*. When *P. cepacia*/pJP4 (fast-growing strain), *P. pickettii*/p712 (slow-growing strain), and *A. paradoxus*/p2811 (slow-growing strain) were inoculated together into 2,4-D mineral medium, the fast-growing strain, as expected, outcompeted the other two slow-growing strains (Fig. 3-B). The similar result was obtained from the mi-

xed culture among *A. eutrophus* JMP134/pJP4, *P. pickettii*/p712, and *A. paradoxus*/p2811 (data not shown), where the plasmid pJP4 was in the different host, *A. eutrophus* JMP134. This result suggests that the plasmid pJP4, which confers fast growing property to its host, is better competitor than plasmids p712 and p2811 in 2,4-D mineral medium. The mixed culture between the fast-growing strains, *P. pseudomallei*/p745 and *P. cepacia*/pJP4, showed that *P. cepacia*/pJP4 multiplied quickly and outcompeted *P. pseudomallei*/p745 (Fig. 3-C). This result was unexpected because, on the basis of the result of the axenic growth experiment, strain *P. pseudomallei*/p745 could have had similar competitiveness as strain *P. cepacia*/pJP 4. This result suggested that relative fitness coefficients derived from the axenic cultures were not always predictive for the mixed culture condition. On the other hand, when p745 was transferred into another host *Alcaligenes* JMP228, transconjugant *Alcaligenes* JMP228/p745 outcompeted *P. cepacia*/pJP4 in the mixed culture (Fig. 3-D). This indicates that the host background, as well as the type of plasmid, plays an important role in determining the competitiveness of the 2,4-D degraders. The better competitiveness of the host *Alcaligenes* JMP228 over *P. cepacia* was also observed in the mixed culture between *Alcaligenes* JMP228/pJP4 and *P. cepacia*/pJP4 (data not shown) and between *Alcaligenes* JMP228/p2811 and *P. cepacia*/p2811 (Fig. 3-A). These results, together with the results from the preceding experiments, showed that hosts *Alcaligenes* JMP228 and probably *P. cepacia* were the better competitors than any other hosts used here and that plasmid pJP4 was more stable and consistently competitive than any other plasmids tested.

This study shows that the plasmid and plasmid-host interactions play an important role in determining the specific growth rate and lag time, respectively, which are key determinants of the competitive outcome. In the future, the fate and competition analysis in natural environment for some of the competitive strains obtained in this study will give valuable information on whether the laboratory studies are predictive of what actually occurs in the open environment.

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