Stimulatory Effect of Glutamine and Pyruvate on Plasmid Transfer between *Pseudomonas* Strains

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The effect of amino acids (glutamine, asparagine, and serine) and organic acids (citrate, malate, and pyruvate) on plasmid transfer was investigated on agar plates using *Pseudomonas* strains differing in physiological status. Counts by culture-dependent and culture-independent methods showed that the presence of organic compounds as the sole nutrient source increased the absolute number of transconjugants. Moreover, the presence of glutamine and pyruvate as the sole nutrient source resulted in a higher transfer frequency. This stimulatory effect of pyruvate was also investigated in short-term conjugation experiments under nutrient-amended conditions with glucose. Compared with glutamine, the presence of pyruvate resulted in a higher transfer frequency after 5 h of conjugation with active cells. The present study confirmed the importance of organic compounds and the physiological status of bacteria to plasmid transfer, and provided evidence that certain organic compounds (glutamine and pyruvate) can stimulate the growth of transconjugants and/or conjugal gene exchange between *Pseudomonas* spp.

**Key words:** plasmid transfer, organic compounds, *Pseudomonas*, glutamine, pyruvate

Lateral gene transfer by plasmid-mediated conjugation is an important mechanism in the acquisition of new genetic traits by bacteria in the natural environment⁴,⁶,³¹. The rhizosphere is a preferred site for bacterial activity in soil, especially *Pseudomonas*, because of root-released organic compounds⁵,³³. The influence of the rhizosphere on plasmid transfer has been investigated in many studies, revealing not only that plasmid transfer is highly stimulated in the rhizosphere but also that there are differences in the kinetics of plasmid transfer among the rhizospheres of different plants²⁷. These observations have suggested the existence of species-specific rhizosphere effects on the stimulation of plasmid transfer and the effects of the composition of root exudates on plasmid transfer should be investigated.

Among the soluble compounds in root exudates, sugars are often reported to be the major components⁵,⁸,¹⁰. However, there is no evidence that sugars play a major role in plant-bacteria interactions⁹,²⁸. Amino and organic acids are also major components in root exudates and have been proposed to be involved in many processes in the rhizosphere⁵,⁸,¹⁵,²⁰,²⁹. In spite of the fact that amino and organic acids are important components of root exudates, their importance in the acquisition of new genetic traits among plant-colonizing bacteria has received little attention. Investigations focused on evaluating the utilization of root-released compounds by bacterial populations and their possible roles in conjugal plasmid transfer are important to advance our knowledge of gene transfer phenomena taking place in natural habitats.

In this study, we addressed the issue of whether the presence of amino and organic acids has an effect on plasmid transfer between *Pseudomonas* strains.
Materials and Methods

Bacterial strains, plasmid, and culture conditions

Conjugation experiments were carried out using Pseudomonas fluorescens RIMD 1615005 as a donor of the plasmid RK2::gfp (a variant of broad-host-range RK2 plasmid ATCC 37125) and Pseudomonas putida ATCC 12633 as a recipient. The RK2::gfp plasmid was constructed by Jorquera et al. and encodes the green fluorescent protein gene and genes imparting resistance to ampicillin, tetracycline, kanamycin (Km), and streptomycin (Sm). Active Pseudomonas cells were obtained from cultures kept over-night in Luria-Bertani (LB) broth at 30°C with shaking at 100 rpm. Starved Pseudomonas cells were obtained after incubation at 4°C for 1 month in phosphate-buffered saline (PBS) without shaking in the dark, as described by Kitaguchi et al.

Active donors were grown in LB broth supplemented with 50 µg ml⁻¹ of Km and 10 µg ml⁻¹ of Sm to ensure maintenance of the RK2::gfp plasmid, while active recipient cells (sensitive to Km and Sm) were grown in LB without the addition of any antibiotic. For starved cells, observations by epifluorescence microscopy did not show any difference in bacterial concentrations as a result of cryptic growth by cellular lysis during the starvation regime and the green fluorescence in donors was taken as an indicator of viability and the stable presence of RK2::gfp as described by Unge et al.

Conjugation experiment in the presence of organic compounds

Conjugations during filter mating were carried out according to the methodology described by Schwaner and Kroer with some modifications. Active and starved parental cells were mated (10⁷ cells of each donor and recipient in 30 µl of sterile 0.9% NaCl solution; donor:recipient=1:1) as follows in triplicate: active donor plus active recipient and starved donor plus starved recipient. Then, mating mixtures were dropped onto cellulose acetate filters (0.45 µm, Advantec., Tokyo, Japan) and the filters were placed on M9 agar plates (minimal medium; Sambrook et al.) in the absence of organic compounds and M9 agar plates supplemented with a sugar (glucose), amino acid (glutamine, asparagine or serine), or organic acid (citrate, malate or pyruvate), as the sole nutrient source at a final concentration of 10 mM each. After incubation at 30°C for 24 h, the filters carrying mated cells were transferred to tubes with 1 ml of sterile 0.9% NaCl solution and cells were dislodged by vortex mixing for 1 min. Finally, the filters were removed aseptically and the bacterial suspensions were used for the estimation of the absolute numbers of the recipient and transconjugant, and transfer frequency (TF) of the plasmid.

Before and after mating, control recipients were placed on M9 agar plates and on M9 agar plates supplemented with 50 µg ml⁻¹ of Km to eliminate the possibility of a spontaneous mutation to Km resistance. Possible changes in the neutral pH of media as a result of supplementation were checked to prevent pH-stress during mating. None of the media prepared showed an abrupt change in pH.

Short-term conjugation experiment

Active and starved parental cells were mated (10⁷ cells of each donor and recipient in 50 µl of sterile 0.9% NaCl solution; donor:recipient=1:1), dropped onto cellulose acetate filters and placed on M9 agar plates supplemented with glucose, or glucose plus organic compounds (glutamine, asparagine, serine, citrate, malate, and pyruvate) at a final concentration of 10 mM each. The filters containing mated cells were incubated at 30°C for 0, 2.5, or 5 h, and bacterial suspensions were obtained as described above and used for the estimation of the absolute numbers of the recipient and transconjugant, and TF of the plasmid.

Estimation of bacterial number and TF of plasmid

Bacterial suspensions were examined by culture-dependent and culture-independent methods for estimation of the absolute numbers of recipients and TF of the plasmid. For the culture-dependent method, bacterial suspensions were diluted and recipients were estimated by plating on M9 agar plates supplemented with 10 mM benzoate (which cannot be metabolized by donor cells) as the sole carbon source. Transconjugants were estimated on M9 agar plates supplemented with 10 mM benzoate and 50 µg ml⁻¹ of Km. The plates were incubated for 4 days at 30°C before colony-forming units (CFU) were counted.

For the culture-independent method, donor, recipient, and transconjugant cells were estimated by a new method described by Jorquera et al. This method involves a combination of direct viable counting (DVC), fluorescence in situ hybridization (FISH), and green fluorescence protein (GFP) gene expression and called the DVC-FISH-GFP method. Briefly, bacterial cells were subjected to DVC with the addition of the parental antimicrobial compounds (50 µg ml⁻¹ of Km and 10 µg ml⁻¹ of Sm), in order to promote selective elongation and/or enlargement of the transconjugants, and incubated at 30°C for 16 h in the dark. After this incubation, the bacterial cells were fixed with paraformaldehyde (8%) at 4°C for 4 h and subjected to fluorescence in
siu hybridization using a *P. putida*-targeted Cy3-labeled probe at 48°C for 2 h. Finally, the cells were directly observed with blue and green excitation under a fluorescence microscope (E-400; Nikon) for viewing GFP-expressing (donor and transconjugant) and Cy3-labeled cells (recipient and transconjugant) and subjected to image analysis and counting as described by Jorquera et al.16).

The TF of the plasmid was expressed as the number of transconjugants relative to the absolute number of recipients (T/R).

**Data analysis**

All counts reported are means of at least three determinations. The differences of bacterial number and TF among different treatments were analyzed with the one-way analysis of variance (ANOVA) and Tukey’s HSD post-hoc test. Probabilities less than or equal to 0.05 were considered significant.

**Results and Discussion**

**Transconjugant growth and TF in the presence of organic compounds**

The number of transconjugants as well as the TF of the plasmid estimated by culture-dependent and culture-independent methods is shown in Figures 1 and 2, respectively. Although no significant difference was detected in the number of transconjugants among the organic compounds in conjugations with active cells (Fig. 1, upper graph, left), more (*p*≤0.05) transconjugants resulted in the presence of organic compounds (from 1.2×10⁶ to 3.3×10⁶ CFU ml⁻¹) than in their absence (2.8×10⁵ CFU ml⁻¹). The number of recipients among organic compounds ranged from 7.1×10⁶ to 2.8×10⁶ CFU ml⁻¹ with lower values in the presence of glutamine (7.1×10⁶ CFU ml⁻¹) and serine (1.7×10⁷ CFU ml⁻¹). The number of recipients in the presence of organic compounds was also significantly high (*p*≤0.05) compared with that in their absence (5.9×10⁵ CFU ml⁻¹). The TF was higher (*p*≤0.05) in the presence of glutamine (2.6×10⁻²) than in the presence of any other organic compound (from 1.0×10⁻² to 1.3×10⁻²) (Fig. 1, lower graph, left).

A similar effect was observed in conjugations with starved parental cells. Significantly more (*p*≤0.05) transconjugants resulted in the presence than absence of organic compounds. A larger (*p*≤0.05) number of transconjugants was observed in the presence of organic acids (from 2.0×10⁵ to 6.8×10⁵ CFU ml⁻¹) than in the presence of amino acids (from 9.1×10⁴ to 3.3×10⁴ CFU ml⁻¹) and absence of organic compounds (4.5×10⁴ CFU ml⁻¹) (Fig. 1, upper graph, right).

The number of recipients among organic compounds ranged from 1.7×10⁶ to 3.7×10⁷ CFU ml⁻¹ with lower values in the presence of glutamine (1.7×10⁶ CFU ml⁻¹) and serine (4.6×10⁶ CFU ml⁻¹). The number of recipients in the presence of organic compounds was also higher (*p*≤0.05) compared with that in their absence (4.3×10⁵ CFU ml⁻¹). A significantly higher (*p*≤0.05) TF was obtained with malate (1.8×10⁻²) and pyruvate (1.7×10⁻²) than with the other organic compounds (from 2.2×10⁻³ to 7.4×10⁻³) (Fig. 1, lower graph, right).

Consistent with the results obtained using the culture-dependent method, the use of the culture-independent method confirmed the increase in the number of transconjugants in the presence of organic compounds (Fig. 2, upper graphs). Conjugations with active and starved parental cells resulted in significantly more (*p*≤0.05) transconjugants in the presence (from 4.5×10⁵ to 1.1×10⁷ cells ml⁻¹) than
absence of organic compounds. Investigations not only have demonstrated that nutrient availability is conducive for plasmid transfer, but also have indicated the relevance of nutrients to the physiological status of bacteria for conjugative gene exchange. Recently Sonawane et al. reported that several strains of _P. fluorescens_ and _P. putida_ rapidly grow on amino acids, even when they are supplied as the sole source of carbon and nitrogen. In addition, Jones and Lugtenberg et al. have suggested that organic acids present in exudates from roots are the nutritional basis for the metabolic activity of _Pseudomonas_ spp. during root colonization and that those organic acids are involved in many processes driven in the rhizosphere. Pseudomonads are capable of using amino and organic acids as a source of nutrients and this resulted in an increase in the absolute number of transconjugants during the conjugation period.

In addition, the high TF obtained in the presence of glutamine suggests that the acquisition of the plasmid might enhance the ability of transconjugants to use glutamine, thereby increasing metabolic activity. The plasmid RK2::gfp is a derivative of RK2 which harbors orphan genes encoding proteins with a high glutamine content (upf34.8 and upf34.4). A complete analysis of the nucleotide sequence of IncPα promiscuous plasmids (R18, R68, RK2, RP1, and RP4) detected a glutamine amido-transf erase class II active site encoded in the plasmids, though its function in specific processes is not yet known. Glutamine amido-transferase is an enzyme that catalyzes the transfer of amide nitrogen to a wide range of substrates, conferring the capacity to use either glutamine or free ammonium. Szpirer et al. have reported that a conjugative plasmid could enter the recipient, express at least a part of the information it carried, and interact with chromosomes, allowing better survival or a more rapid growth of transconjugants. The possibility of glutamine-related genes and interaction of RK2::gfp with the chromosomes of recipients might become clear with further experiments.

It is necessary to mention the discrepancy obtained in the absolute number of recipient cells and transconjugants and/or TF between culture-dependent and culture-independent methods (especially in the absence of organic compounds). Arana et al. have demonstrated that a fraction of non-cultur able recipients are able to receive and express plasmids via conjugation processes and form viable but non-culturable transconjugant cells which are overlooked by conventional plate-counting methods. In this context, oligothrophic or nutrient-limited conditions during conjugation might induce recipient and/or transconjugant cells to enter into a viable but non-culturable state. This low metabolic activity would
mean that the culture-dependent approach underestimates the absolute number of bacterial cells. Hence, the underestimation of recipients in the absence of organic compounds might have resulted in a higher TF by the culture-dependent method compared with that obtained by the culture-independent method.

In conjugations with starved cells, the large number of transconjugants and high TF in the presence of pyruvate (detected with both methods) suggest that this organic acid produced a stimulatory effect on plasmid transfer between starved cells. As mentioned above, an organic acid such as pyruvate might not only be used efficiently as a nutrient source by bacterial cells enhancing the physiological status of bacteria but also be involved in the promotion of conjugation events. Although the use of culture-independent methods allow an accurate estimation of recipient and transconjugant numbers at the single cell level compared with culture-dependent methods, one cannot differentiate between descendants of primary transconjugants and new transconjugants that appeared during the conjugation period. Sørensen et al. indicated that conventional methods for detecting plasmid transfer do not distinguish between increased numbers of transfer events and post-transfer detection (clonal expansion of parental cells and transconjugants during long-term conjugation). This limitation cannot be completely eliminated but it might be reduced by performing conjugation for a short time.

Transconjugants were recovered in the absence of organic compounds with starved parental cells. This result coincides with previous observations on plasmid transfer between starved or non-growing bacteria under nutrient-depleted conditions which have indicated that plasmid transfer occurs independently of bacterial growth and that a minimum level of metabolic activity seems to be necessary.

Transconjugant growth and TF in short-term conjugation

Given the conjugation in the presence of organic compounds and in order to elucidate the effect of amino and organic acids on the transfer frequency of the plasmid, short-term conjugation experiments under nutrient-amended conditions were carried out. Short-term conjugation was used to prevent an increase in the number of recipients and transconjugants during the conjugation period, reducing the effect of the absolute number of recipients on TF and the possibility that the counts include both descendants of the primary transconjugant and new transconjugants. Glucose was employed as a carbon source because this compound is easily metabolizable by bacteria and maintained the physiological activity of the bacteria in this short-term conjugation experiment.

The culture-dependent method did not detect transconjugants after 2 h of conjugation. By using the culture-independent method, a small number of transconjugants were observed but no significant difference was detected in transconjugants among the organic compounds after 2 h of conjugation (data not shown).

After 5 h of conjugation with active parental cells, the culture-dependent method showed an increase (p≤0.05) of transconjugants in the presence of glucose plus pyruvate (5.1×10³ CFU ml⁻¹) compared with glucose alone (7.0×10² CFU ml⁻¹) and glucose plus glutamine (1.5×10² CFU ml⁻¹) (Fig. 3, upper graph, left). The number of recipients ranged from 3.8×10⁶ to 5.0×10⁶ CFU ml⁻¹ and a significantly higher (p≤0.05) TF was also obtained in the presence of glucose plus pyruvate (1.1×10⁻³) compared with in the presence of glucose alone (1.1×10⁻⁴) and glucose plus glutamine (3.5×10⁻⁵) (Fig. 3, lower graph, left).

A similar tendency was observed by using the culture-

![Fig. 3. Number of transconjugants (upper graph) and transfer frequency of the plasmid (lower graph) estimated by culture-dependent (A) and culture-independent (B) methods. Data was obtained after 5 h of conjugation with active parental cells. Horizontal axis: M9 agar supplemented with glucose (G), glucose plus glutamine (GGI), and glucose plus pyruvate (GP). Error bars indicate the standard deviation.](image-url)
independent method. The number of transconjugants was greater (p≤0.05) in the presence of glucose plus pyruvate (8.7×10^6 cells ml^-1) than with glucose alone (5.6×10^6 cells ml^-1) or glucose plus glutamine (2.3×10^6 cells ml^-1) (Fig. 3, upper graph, right). The number of recipients ranged from 5.0×10^7 to 1.3×10^8 cells ml^-1 and a significantly higher (p≤0.05) TF was obtained in the presence of glucose plus pyruvate (7.2×10^7) compared with glucose alone (1.2×10^7) and glucose plus glutamine (1.9×10^7) (Fig. 3, lower graph, right).

The metabolic process in bacteria generally begins with the hydrolysis of large molecules (proteins, polysaccharides, and lipids) in the external cellular environment by specific exoenzymes. The small subunit molecules produced (amino acids, monosaccharides, and glycerol) are transported across the cell membrane into the cytoplasm and converted by one or more pathways to one common universal intermediate, pyruvate. From pyruvate, the carbons may be channeled toward energy production or the synthesis of new compounds\(^1\). In this context, the plasmid RK2 encodes two genes (incC and korB) that belong to the parA and parB protein families. parB is a DNA-binding protein that interacts with parA whose ATPase activity (energy supply) is essential to the segregation process. Both are responsible for the coordinated regulation of operons encoding replication, transfer, and inheritance stable maintenance functions of the plasmid in hosts\(^1\).

This important role of pyruvate in the metabolism of bacteria might explain the faster increase in the number of transconjugants during conjugation compared with that obtained with glucose alone, which must be converted to pyruvate before entering the metabolic pathway. In relation to TF, pyruvate might be more easily or more quickly channeled toward energy production compared with glucose and other organic compounds, enhancing the plasmid transfer process.

Conjugations with starved cells after 5 h did not show a significant difference in the absolute number of transconjugants or TF between the culture-dependent and culture-independent methods (data not shown).

Diverse investigations have established that plasmid transfer among bacteria is influenced by diverse biotic and abiotic factors, including the species involved, nutrient availability, physiological status of bacteria, distribution and density of cells, temperature, pH, selective pressure, and chemical compounds\(^3,7,13,14,19,22,25\). The present study not only confirmed the importance of the presence of organic compounds (naturally found in the environment) and physiological status of bacteria to plasmid transfer, but also provided evidence that certain organic compounds (specifically glutamine and pyruvate) can stimulate the growth of transconjugants and/or conjugal gene exchange events during under different physiological conditions. These stimulatory effects would govern the acquisition, spread, and occurrence of plasmids among bacterial populations in the environment, specifically in rhizospheres.

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