FEMS Microbiology Ecology 51 (2004) 1–8

www.fems-microbiology.org

The enhancement of conjugal plasmid pBHR1 transfer between bacteria in the presence of extracellular metabolic products produced by *Microcystis aeruginosa*

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Received 9 February 2004; received in revised form 24 June 2004; accepted 8 July 2004

First published online 2 August 2004

Abstract

Conjugal plasmid transfer from *Escherichia coli* S17-1 (pBHR1) to *Pseudomonas stutzeri* was investigated in the presence of a cyanophyta *Microcystis aeruginosa*. The plasmid transfer frequency increased with higher densities of *M. aeruginosa*. The extracellular metabolic products (EMPs) from *M. aeruginosa* were found to enhance the plasmid transfer between bacteria. Furthermore, the plasmid transfer frequency in medium containing EMPs was significantly higher than that in culture medium with or without glucose. These results suggest that *M. aeruginosa* enhances conjugal plasmid transfer between bacteria through its EMPs, and that identity of the carbon source is an important factor affecting conjugal plasmid transfer in aquatic environments.

Keywords: Conjugal plasmid transfer; *Microcystis aeruginosa*; Extracellular metabolic products; Aquatic environments

1. Introduction

Bacteria can obtain new genetic characters through horizontal gene transfer. Conjugal gene transfer is an important mechanism of gene transfer between bacteria. To clarify the possibility that conjugal gene transfer can occur in the natural environment, transfer frequencies have been investigated in various terrestrial [1–4] and aquatic [5–12] environments.

Studies on conjugal plasmid transfer in aquatic environments, using sterilized environmental water microcosms [5,6,8,12], have shown that plasmid transfer does occur. However, one important difference between sterilized aquatic environments and natural aquatic environments is the existence of other organisms. Both donor and recipient bacteria may interact with numerous microorganisms through, for example, microbial loops in the natural aquatic environment [13]. It is possible that the presence of other microorganisms might influence the conjugal gene transfer process. It has been suggested that the presence of microbial communities leads to a reduced conjugal plasmid transfer frequency [8]; however, the reasons for this are unclear.

Organic substrates and water temperature are considered important chemical and physical factors leading to enhancing conjugal plasmid transfer [7,14]. These factors also affect bacterial growth and activity. In aquatic ecosystems, extracellular metabolic products (EMPs) from phytoplankton, which contain a mixture of

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0168-6496/$22.00
doi:10.1016/j.femsec.2004.07.003

organic and inorganic compounds, provide important substrates for the growth of heterotrophic bacteria [13,15]. Therefore, we hypothesized that the presence of phytoplankton can potentially enhance conjugal plasmid transfer through the production of EMPs.

Microcystis aeruginosa is a bloom-forming cyanophyta that occurs in many eutrophic and hypertrophic lakes and ponds in both tropical and temperate regions. During bloom-formation periods, *M. aeruginosa* can reach cell densities ranging from 10^5 to 10^7 cells ml^{-1} [16–18] and following the formation of surface scums, to as high as 10^9 cells ml^{-1} [19]. The production of extracellular organic matter (EOM) by *M. aeruginosa* has been reported to reach 1.410 mgC l^{-1} h^{-1} [20]. More than 90% of the EOM produced by *M. aeruginosa* is in the low molecular size range (<700 Da) and is considered a suitable carbon source for bacterial use [21]. Therefore, it is possible that the presence of a higher density of *M. aeruginosa* might increase the frequency of conjugal plasmid transfer between heterotrophic bacteria.

The aim of this study was to investigate the effect of *M. aeruginosa* on conjugal plasmid transfer. We carried out experiments to determine whether the presence of *M. aeruginosa* enhanced the conjugal plasmid transfer from donor to recipient, whether the EMPs produced by *M. aeruginosa* increased the frequency of conjugal plasmid transfer, and whether EMPs or glucose are a more suitable labile carbon source for the substrates of enhancing conjugal plasmid transfer between bacteria.

2. Materials and methods

2.1. Organisms and their preparation

A streptomycin-resistant strain of *Escherichia coli* S17-1 [22] was used as a donor in this study. The pBHR1 plasmid [23,24] was transferred into *E. coli* S17-1 by the SEM method [25]. The pBHR1 is a mobilization plasmid coding for kanamycin resistance and was obtained from the Molecular Biologische Technologie, Goettingen, Germany. Plasmid pBHR1 was made by replacing the fragment containing the *mob* gene of pBBR1CM with the homologous fragment of pBBR122. The Inc P type helper conjugative plasmid is needed to transfer pBHR1 [23]. In this study, plasmid RP4 (Inc P) is integrated within the chromosome of *E. coli* S17-1 [22]. Therefore, pBHR1 transfer from transconjugant to recipient does not occur. A nalidixic acid-resistant and non-naturally transformed strain of *Pseudomonas stutzeri* (ATCC17588) was used as the recipient strain for the pBHR1 plasmid. An axenic, unicellular strain of *M. aeruginosa* (NIES-298) was obtained from the National Institute for Environmental Studies (NIES), Environmental Agency, Tsukuba, Japan.

We used the following procedures in preparing organisms for all of experiments. Bacteria used to inoculate the cultures were stored at −80 °C in LB broth with 20% (v/v) glycerol. The donor *E. coli* S17-1 (pBHR1) was grown overnight on LB agar containing both streptomycin (50 μg ml^{-1}) and kanamycin (100 μg ml^{-1}). The recipient bacterium (*P. stutzeri*) was grown overnight on LB agar containing nalidixic acid (20 μg ml^{-1}). After overnight culture, *E. coli* S17-1 (pBHR1) was inoculated into 500 ml of LB broth containing both streptomycin (50 μg ml^{-1}) and kanamycin (100 μg ml^{-1}) and *P. stutzeri* was inoculated into 500 ml of LB broth containing nalidixic acid (20 μg ml^{-1}). These bacteria were grown at 37 °C with shaking (150 rpm) for 14 h. Each of the bacterial suspensions was centrifuged ($5000 \times g$, 10 min) and washed five times, then resuspended in CBY100 medium (i.e., 100 mg l^{-1} yeast extract in CB medium [26]). CBY100 medium was used for all of the mating experiments. *M. aeruginosa* was cultured for 3 weeks in flasks containing 2 l of MA medium [26] with aeration, at a light intensity of 60 μE m^{-2} s^{-1} on a 12:12 h light:dark cycle at 25 °C. When the cell density of *M. aeruginosa* reached approximately 1×10^7 cells ml^{-1}, the cells were collected by centrifugation ($4000 \times g$, 10 min), washed three times, then resuspended in fresh CBY100 medium.

2.2. Conjugation experiment in the presence of M. aeruginosa

To investigate the effect of *M. aeruginosa* on conjugal plasmid transfer, we carried out triplicate experiments using three different cell densities of *M. aeruginosa*. Erlenmeyer flasks containing 150 ml of CBY100 medium were inoculated with *M. aeruginosa* at two different final cell densities, 6.9×10^5 and 1.4×10^7 cells ml^{-1}, respectively; a third flask was not inoculated with *M. aeruginosa*. Both *E. coli* S17-1 (pBHR1) and *P. stutzeri* were added to the three flasks at final densities of 1×10^7 CFU ml^{-1}. The flasks were cultured at a light intensity of 60 μE m^{-2} s^{-1} on a 12:12 h light:dark cycle and at 30 °C with shaking (70 rpm). Samples were collected from each of the flasks at 0, 6, 12, and 30 h after inoculation, and the cell densities of *E. coli* S17-1 (pBHR1), *P. stutzeri*, and the transconjugant *P. stutzeri* (pBHR1) were enumerated. The transfer of pBHR1 from *E. coli* S17-1 to *P. stutzeri* was detected by the development of kanamycin resistance. Samples taken from the cultures (100 μl) were spread on LB agar containing both streptomycin (50 μg ml^{-1}) and kanamycin (100 μg ml^{-1}) for *E. coli* S17-1 (pBHR1), nalidixic acid (20 μg ml^{-1}) for *P. stutzeri*, or both nalidixic acid (20 μg ml^{-1}) and kanamycin (100 μg ml^{-1}) for the transconjugant. The plates were incubated at 37 °C for 40 h to allow colony formation.

2.3. Effect of EMPs from M. aeruginosa on conjugal plasmid transfer

To investigate the effect of EMPs from *M. aeruginosa* on conjugal plasmid transfer, four treatments were prepared in triplicate flasks containing 100 ml of CBY100 medium. The four treatments were prepared as follows and designated by a lettered flask: (1) No *M. aeruginosa* added (C flask); (2) EMPs from *M. aeruginosa* (E flask); (3) heat-treated *M. aeruginosa* cell particles at a final density of 1.0×10^7 particles ml^{-1} (P flask); and (4) viable *M. aeruginosa* cultures at a final density of 1.0×10^7 cells ml^{-1} (M flask). The E flask containing EMPs from *M. aeruginosa* was prepared using the following method: Erlenmeyer flasks containing 100 ml of CBY100 medium were inoculated with *M. aeruginosa* at a final density of 1.0×10^7 cells ml^{-1} and incubated for 24 h at a light intensity of 60 μE m^{-2} s^{-1} on a 12:12 h light:dark cycle and at 30 °C with shaking (70 rpm). After incubation for 24 h, the cells were removed by centrifugation ($4000 \times g$, 10 min) and the supernatant was filtered through a glass-fiber filter with a pore size of 0.3 μm (GF-75, Advantec, Japan), which was heated at 570 °C for 3 h before use. The P flask, which contained the heat-treated *M. aeruginosa* particles, was prepared by heating the suspended *M. aeruginosa* cells in CBY100 media at 70 °C for 1 h. The suspension was then centrifuged, and the pellet was washed three times with CBY100 medium.

All four treatment flasks were inoculated with 1×10^7 CFU ml^{-1} of *E. coli* S17-1 (pBHR1) and 1×10^7 CFU ml^{-1} of *P. stutzeri*. After an incubation period of 24 h at a light intensity of 60 μE m^{-2} s^{-1} on a 12:12 h light:dark cycle and at 30 °C with shaking (70 rpm), the numbers of donor, recipient, and transconjugant cells were enumerated according to the method described in Section 2.2.

2.4. Comparison of conjugal plasmid transfer frequency between EMPs and glucose

We compared the ability of EMPs from *M. aeruginosa* to influence conjugal plasmid transfer with that of glucose as an available carbon source. For this experiment, a glucose solution (10.2 gC l^{-1}), which had been passed through a 0.22 μm pore size PVDF filter (Vacuum Filtration System, Iwaki, Japan), was added to CBY100 medium at final concentrations of 20.6, 72.1, and 123.6 mgC l^{-1}. CBY100 medium with and without EMPs from *M. aeruginosa* were also prepared, as described in Section 2.3. All kinds of medium were each inoculated with *E. coli* S17-1 (pBHR1) and *P. stutzeri* at an initial density of 1×10^7 CFU ml^{-1}, respectively. After incubation for 24 h at 30 °C in the dark with shaking (70 rpm), the numbers of *E. coli* S17-1 (pBHR1), *P. stutzeri*, and transconjugant were counted using the method described in Section 2.2. The concentration of EMPs was estimated by measuring the amount of dissolved organic carbon using a TOC-5000A total organic carbon analyzer (Shimadzu, Japan).

2.5. Calculation of transfer frequency and statistical analyses

The frequency of conjugal plasmid transfer was represented as the number of the transconjugants relative to the number of *E. coli* S17-1 (pBHR1) (T/D). Statistical tests were carried out using the StatView program developed for Macintosh computers. The Friedman test was used to analyze the differences in the number of the transconjugants and transfer frequencies between treatments over time. A one-way analysis of variance (ANOVA) with Fisher's PLSD test was used to analyze the differences in the numbers of transconjugants and the values of transfer frequencies between treatments at each sampling time. A significance level of 0.05 was used for all statistical analyses.

3. Results

3.1. Conjugal plasmid transfer in the presence of M. aeruginosa

Cell densities of donor *E. coli* S17-1 (pBHR1) remained between 1.0×10^7 and 2.7×10^7 CFU ml^{-1} throughout this experiment in all treatment flasks. However, in the flasks with either a low density (6.9×10^5 cells ml^{-1}) or absence of *M. aeruginosa*, recipient *P. stutzeri* reached densities from 1.1 to 2.0×10^8 CFU ml^{-1} within 6 h. In flasks containing a high density (1.4×10^7 cells ml^{-1}) of *M. aeruginosa*, the cell density of *P. stutzeri* remained low (4.8-8.0×10^7 CFU ml^{-1}) over the entire experimental period. The number of transconjugants as well as the frequency of plasmid transfer is shown in Table 1. At each of the sampling times, there were significant differences between treatments with respect to the number of transconjugants. This result was demonstrated by ANOVA ($p = 0.0044$ at 6 h, $p = 0.0033$ at 12 h, and $p = 0.0011$ at 30 h). Using the same statistical method, we also detected significant differences between treatments with respect to plasmid transfer frequencies at each of the sampling times ($p = 0.0028$ at 6 h, $p = 0.0084$ at 12 h, and $p = 0.0002$ at 30 h). Both the number of transconjugants produced and the transfer frequencies with time were found to increase significantly with increasing cell density of *M. aeruginosa* (Friedman test, $p = 0.0003$ and 0.0001, respectively).

Table 1
Number of transconjugant and transfer frequency co-cultured with and without *M. aeruginosa*

	Time (h)	Transconjugant[a] (CFU ml^{-1})	Transfer frequency[a] (T/D)
Flask without *M. aeruginosa*	6	$2.2 \pm 0.7 \times 10^{2}$	$1.3 \pm 0.5 \times 10^{-5}$
	12	$1.2 \pm 0.1 \times 10^{3}$	$4.5 \pm 0.1 \times 10^{-5}$
	30	$5.7 \pm 0.7 \times 10^{3}$	$2.9 \pm 0.6 \times 10^{-4}$
Flask with low density of *M. aeruginosa* (6.9×10^{5} cells ml^{-1})	6	$3.7 \pm 1.3 \times 10^{2}$	$2.2 \pm 0.7 \times 10^{-5}$
	12	$1.4 \pm 0.1 \times 10^{3}$	$6.2 \pm 1.3 \times 10^{-5}$
	30	$8.8 \pm 1.3 \times 10^{3}$	$4.2 \pm 0.5 \times 10^{-4}$
Flask with high density of *M. aeruginosa* (1.4×10^{7} cells ml^{-1})	6	$6.6 \pm 1.1 \times 10^{2}$	$4.5 \pm 0.8 \times 10^{-5}$
	12	$1.9 \pm 0.2 \times 10^{3}$	$2.2 \pm 0.7 \times 10^{-4}$
	30	$1.8 \pm 0.3 \times 10^{4}$	$1.5 \pm 0.3 \times 10^{-3}$

[a] Values present the mean and standard deviation from triplicates.

3.2. Effects of EMPs from M. aeruginosa on conjugal plasmid transfer

In this experiment, we used the following four treatments flasks: (1) CBY100 medium with no *M. aeruginosa* (C flasks); (2) CBY100 medium with EMPs from *M. aeruginosa* (E flasks); (3) CBY100 medium with heat-treated *M. aeruginosa* cell particles at a final density of 1.0×10^{7} particles ml^{-1} (P flasks); and (4) CBY100 medium with viable *M. aeruginosa* at a final density of 1.0×10^{7} cells ml^{-1} (M flasks). After a 24 h incubation period, cell densities of *E. coli* S17-1 (pBHR1) remained close to initial density values ($1.0 \pm 0.1 \times 10^{7}$, $1.0 \pm 0.1 \times 10^{7}$, $1.1 \pm 0.1 \times 10^{7}$, and $9.5 \pm 0.6 \times 10^{6}$ CFU ml^{-1}, respectively). After 24 h, *P. stutzeri* grew to $6.5 \pm 1.2 \times 10^{7}$ CFU ml^{-1} in C flasks, $7.1 \pm 1.0 \times 10^{7}$ CFU ml^{-1} in E flasks, $8.4 \pm 0.6 \times 10^{7}$ CFU ml^{-1} in P flasks, and $8.3 \pm 1.1 \times 10^{7}$ CFU ml^{-1} in M flasks. The numbers of transconjugants in the C and P flasks were $4.3 \pm 0.4 \times 10^{2}$ and $4.4 \pm 0.7 \times 10^{2}$ CFU ml^{-1}, respectively (Fig. 1(a)). In the E and M flasks, the numbers of transconjugants ($2.2 \pm 0.1 \times 10^{3}$ CFU ml^{-1} and $1.9 \pm 0.0 \times 10^{3}$ CFU ml^{-1}, respectively) were five times higher than in the C flasks (Fig. 1(a)). The results of an ANOVA with Fisher's PLSD test showed significant differences between the numbers of transconjugants in the C and E flasks ($p < 0.0001$), C and M flasks ($p < 0.0001$), P and E flasks ($p < 0.0001$), P and M flasks ($p < 0.0001$), and E and M flasks ($p = 0.0105$). No significant difference was detected between the C and P flasks. The transfer frequencies were as follows: $4.3 \pm 0.7 \times 10^{-5}$ in the C flasks, $2.2 \pm 0.1 \times 10^{-4}$ in the E flasks, $4.1 \pm 0.6 \times 10^{-5}$ in the P flasks, and $1.9 \pm 0.2 \times 10^{-4}$ in the M flasks (Fig. 1(b)). Significant differences were detected between the values obtained for transfer frequencies in the C and E flasks ($p < 0.0001$), C and M flasks ($p < 0.0001$), P and E flasks ($p < 0.0001$), and P and M flasks ($p < 0.0001$). No significant difference was detected in the values for transfer frequencies between the C and P flasks or between the E and M flasks.

3.3. Comparison of the EMPs from M. aeruginosa with glucose as a carbon source

After 24 h, *E. coli* S17-1 (pBHR1) and *P. stutzeri* reached densities of $5.9 \pm 0.7 \times 10^{7}$ and $9.5 \pm 1.1 \times 10^{7}$ CFU ml^{-1} in CBY100 medium, $5.8 \pm 0.6 \times 10^{7}$ and $1.0 \pm 0.0 \times 10^{8}$ CFU ml^{-1} in CBY100 medium with 20.6 mgC l^{-1} of glucose, $6.2 \pm 0.7 \times 10^{7}$ and $1.1 \pm 0.1 \times 10^{8}$ CFU ml^{-1} in CBY100 medium with 72.1 mgC l^{-1} of glucose, $5.8 \pm 0.4 \times 10^{7}$ and $1.1 \pm 0.1 \times 10^{8}$ CFU ml^{-1} in CBY100 medium with 123.6 mgC l^{-1} of glucose, and $4.5 \pm 0.3 \times 10^{7}$ and $9.9 \pm 0.9 \times 10^{7}$ CFU ml^{-1} in CBY100 medium containing EMPs from *M. aeruginosa* (73.0 mgC l^{-1}). The numbers of transconjugants in the CBY100 medium in the presence of glucose concentrations of 20.6, 72.1, and 123.6 mgC l^{-1} were $5.7 \pm 1.2 \times 10^{2}$, $6.5 \pm 0.9 \times 10^{2}$, and $5.2 \pm 1.7 \times 10^{2}$ CFU ml^{-1}, respectively (Fig. 2(a)). There were no statistical differences among the numbers of transconjugants at different glucose concentrations and the number of transconjugants in the CBY100 medium ($9.8 \pm 0.9 \times 10^{2}$ CFU ml^{-1}). However, the number of transconjugants in the CBY100 medium containing EMPs from *M. aeruginosa* ($6.5 \pm 0.8 \times 10^{3}$ CFU ml^{-1}) was significantly higher than those in CBY100 medium with and without glucose. Using an ANOVA with Fisher's PLSD, a p value of <0.0001 was obtained between that in CBY100 medium with the EMPs and in other treatments. Similarly, the transfer frequency in the CBY100 medium containing EMPs from *M. aeruginosa* was significantly higher than the transfer frequencies in CBY100 medium with and without glucose (Fig. 2(b)), as confirmed by ANOVA with Fisher's PLSD ($p < 0.0001$).

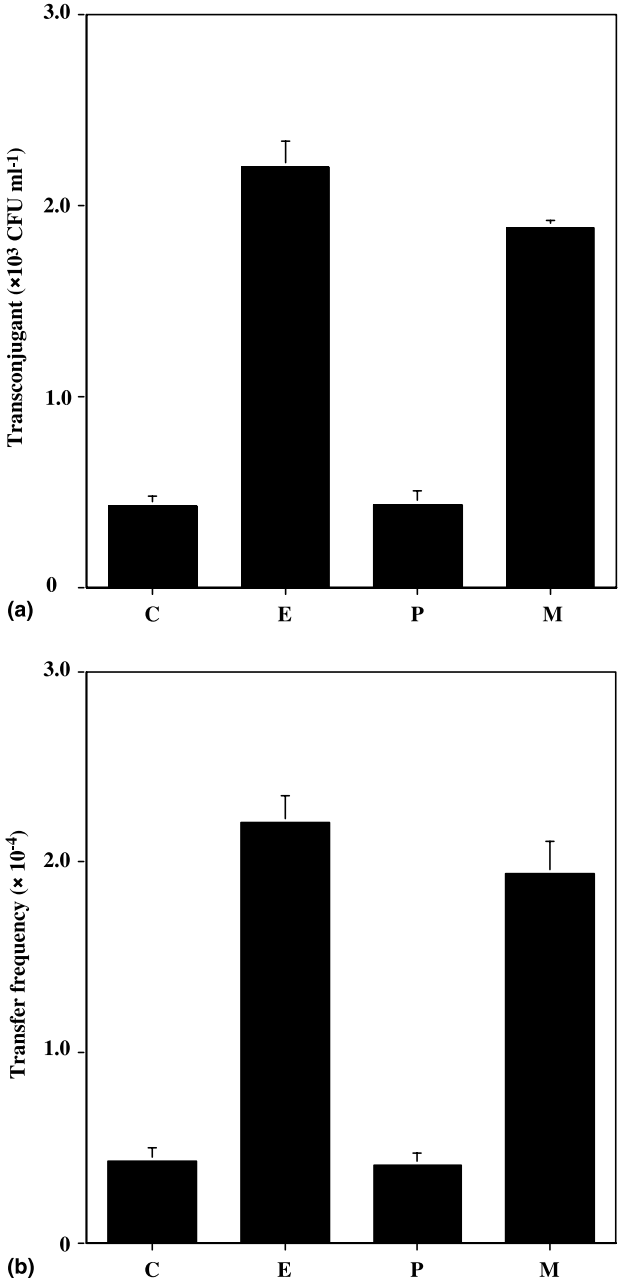

Fig. 1. Number of transconjugant (a) and transfer frequency (b) in several experimental conditions. In horizontal axis, C, E, P and M indicate each experimental condition which is CBY100 medium, CBY100 medium with EMPs of *M. aeruginosa*, CBY100 medium with boiled cell particles of *M. aeruginosa* and CBY100 with alive *M. aeruginosa* cells, respectively. Each value represents mean and standard deviation from triplicates.

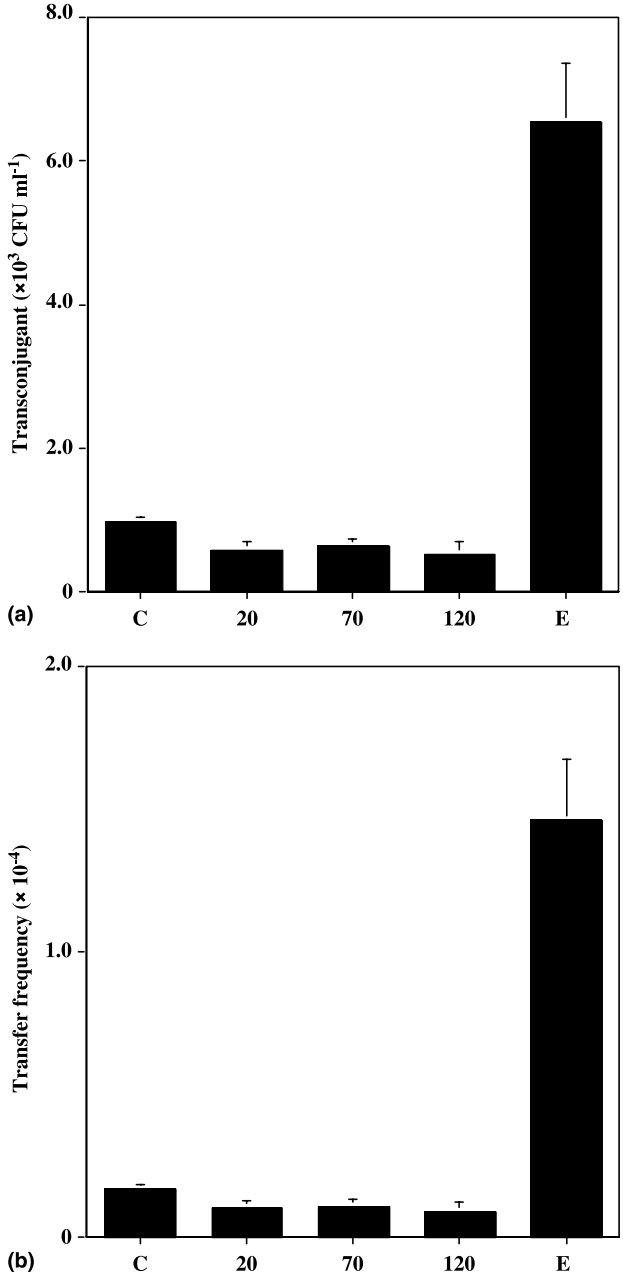

Fig. 2. Comparison EMPs of *M. aeruginosa* with glucose as carbon source. In horizontal axis, C and E indicate each experimental condition which is CBY100 medium and CBY100 medium with EMPs of *M. aeruginosa*. Numbers of 20, 70 and 120 at horizontal axis indicated in the concentration of the glucose (final concentration; 20.6, 72.1 and 123.6 mgC l^{-1}, respectively) added into CBY100 medium. In these culture medium, number of transconjugant (a) and transfer frequency (b) are shown. Each value presents mean and standard deviation from triplicates.

4. Discussion

This study is the first report showing that the EMPs produced by *M. aeruginosa* can enhance conjugal plasmid transfer from *E. coli* S17-1 (pBHR1) to *P. stutzeri* (Table 1 and Fig. 1). In our preliminary experiment using tetrazolium salt, sodium 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) [27,28], the electron transport system (ETS) activity of *E. coli* S17-1 (pBHR1) in CBY100 medium with EMPs was significantly higher than in CBY100 medium alone (data not shown). It has been suggested that bacterial metabolic

activity correlates positively with the conjugal plasmid transfer frequency [3], so the increased ETS activity that was observed in CBY100 medium with EMPs might be a key mechanism in the high conjugal plasmid transfer in the flasks containing either *M. aeruginosa* or its EMPs. On the other hand, it is known that the amount of space available for bacteria to colonize, except for factors that enhance bacterial activity, is important for promoting conjugal gene transfer [29]. In the first experiment (Table 1), the higher densities of *M. aeruginosa* cells might have produced a greater number of sites for potential bacterial colonization. The results shown in Fig. 1 indicate that non-active cell particles (i.e., heat-treated *M. aeruginosa* cells) did not affect the number of transconjugants produced or the transfer frequency. The strain of *M. aeruginosa* used in this study is unicellular, consisting of spherical cells with diameters ranging from 2 to 3 μm. We did not observe any aggregation of *M. aeruginosa* cells under the microscope. Therefore, *E. coli* S17-1 (pBHR1) and *P. stutzeri* may not be able to adhere to viable *M. aeruginosa* cells. Hence, the enhancing effect of *M. aeruginosa* on conjugal plasmid transfer was most likely caused by the presence of its EMPs.

The EMPs from *M. aeruginosa* NIES-298 cultured in CB medium can be classified into five types of organic components (hydrophobic acids, hydrophobic neutrals, hydrophilic acids, hydrophilic neutrals, and hydrophilic bases) [30]. However, more detailed information on the chemical composition of these EMPs is not currently known. Therefore, we could not study the particular chemical compounds that are known to increase the conjugal plasmid transfer frequency. However, there are several studies showing that the extracellular organic compounds produced by *M. aeruginosa* have small molecular sizes (<700 Da) and are readily available for bacterial metabolism [21]. It has also been demonstrated that the presence of these compounds can increase the activity (estimated by thymidine uptake [31]) and growth of wild bacteria in lake ecosystems [32]. According to previous studies and our preliminary experiment, it is likely that by increasing bacterial activity EMPs may be an enhancing factor in conjugal plasmid transfer.

We also compared the enhancing effect of the EMPs from *M. aeruginosa* on conjugal plasmid transfer from *E. coli* S17-1 (pBHR1) to *P. stutzeri* using glucose as a labile carbon source. Although, we detected an enhancing effect on conjugal plasmid transfer in flasks with EMPs (73.0 mgC l^{-1}) from *M. aeruginosa*, a similar enhancing effect was not found in flasks supplied with glucose (20.6–123.6 mgC l^{-1}) (Fig. 2). This indicates that the EMPs from *M. aeruginosa* is more effective to the enhancing conjugal plasmid transfer rather than glucose. Therefore, it is possible that the identity of the carbon source, rather than the quantity, is more important as a factor affecting conjugal plasmid transfer.

In our experiments, the production of *M. aeruginosa* EMPs was estimated by analyzing the extracellular dissolved organic carbon (EOC), which is a component of EMPs. The EOC production rate of *M. aeruginosa* at a cell density of 1.0×10^7 cells ml^{-1} was found to be 73.0 mgC l^{-1} day^{-1}. This value is much higher than that used in other laboratory experiments (0.002 mgC l^{-1} day^{-1}) *and* obtained at a *M. aeruginosa* density of 10^5 cells ml^{-1} [21]. The quantities of EOC containing EMPs might vary between the strain, growth phase, and culture conditions of *M. aeruginosa*. In natural aquatic environments, a value of 1.410 mgC $l^{-1}h^{-1}$ has been reported in a previous study as the EOC production rate of *M. aeruginosa* that dominated a hypereutrophic lake surface [20]. For a *M. aeruginosa* hyperscum, 280–680 mgC l^{-1} of dissolved organic carbon has been reported for a hypereutrophic lake [19]. Thus, in eutrophic aquatic environments where *M. aeruginosa* blooms occur, it is possible that the concentrations of EMPs from *M. aeruginosa* reach the values detected in our experiment.

In this study, we counted the donor, recipient, and transconjugants using selection plates and calculated the transfer frequency from the numbers of donors and transconjugants. Two problems might occur when using this method; one is that mating can occur on the selection plate, if the concentration of antibiotics is too low or if bacterial density is extremely high. We carried out an additional experiment to examine the possibility of mating on the selection plate. Both *E. coli* S17-1 (pBHR1) (3.5×10^7 CFU ml^{-1} at final density) and *P. stutzeri* (1.6×10^8 CFU ml^{-1} at final density) were added to CBY100 medium, CBY100 medium with the EMPs, and CBY100 medium with glucose. We counted the number of transconjugants using selection plates at 0 and 24 h. In this experiment, although we detected the transconjugant colonies in the 24 h sample, no transconjugant colonies were detected in the 0 h samples (data not shown). These results suggest that mating did not occur on the selection plate in our experiments. The second possible problem is that the number of transconjugant colonies counted might include both the descendants of the primary transconjugant and new transconjugants. This possibility cannot be completely ruled out. However, the density of *P. stutzeri* in the flasks in which high frequencies of plasmid transfer depended on a high number of transconjugants (Table 1 and Figs. 1 and 2) did not exceed the density in the other flasks. When it assumes that *P. stutzeri* and transconjugant *P. stutzeri* (pBHR1) grew at the same rate, it is unlikely that descendants of the primary transconjugant caused the high number of transconjugants in those flasks. Moreover, since pBHR1 contains only antibiotic-resistant, *mob* and *ori* genes, which are not responsible for the growth of the transconjugants in the experimental conditions, it is unlikely that the transconjugant had a growth advantage as compared to *P. stutzeri*. There-

fore, we concluded the significantly higher number of transconjugant colonies and of transfer frequencies observed in this study as rising from conjugal plasmid transfer.

Many studies have been carried out to investigate conjugal plasmid transfer between bacteria in the natural environment [33,34]. In previous studies, the potential occurrence of conjugal plasmid transfer in natural aquatic environments has been investigated using sterilized environmental water samples [5,6,8,12], and it has been shown that natural microbial communities in the lake epilimnion suppressed conjugal plasmid transfer [8]. Conversely, our results demonstrate that conjugal plasmid transfer is enhanced in the presence of *M. aeruginosa*. On the other hand, it has been pointed out that the amount of organic carbon is an important enhancing factor for conjugal gene transfer [14]. However, we found that EMPs from *M. aeruginosa* enhanced conjugal plasmid transfer more than glucose as a labile carbon source in the laboratory experiment. Based on our findings, the presence of *M. aeruginosa* bloom may indicate frequent conjugal plasmid transfer among bacteria in the aquatic environment. In order to know whether this is true or not, it is necessary to clarify the effects of other biological, physical and chemical factors on conjugal gene transfer in the natural environments, where *M. aeruginosa* forming its bloom.

Acknowledgements

We thank Profs. M. Nasu and G. Endo and Dr. N. Yamaguchi for their support for carbon analysis and their useful discussion. This works was supported in part by Grant-in-Aid for Scientific Researches 'Analysis of gene transfer and expression mechanisms in aquatic ecosystems using Aquatron', 'Microbiologically environmental assessment in Southeast Asia' and 'Comparative analysis of gene transfer processes in aquatic ecosystems using Aquatron', the grant for the Biodiversity Research of the 21st century COE(A14), and a Grant-in-Aid from The Asahi Glass Foundation 'Analysis of gene kinetics in natural aquatic ecosystems'.

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