Biotic and Abiotic Factors Affecting Plasmid Transfer in Escherichia coli Strains

AURORA FERNANDEZ-ASTORGA,1* ALICIA MUELA,2 RAMÓN CISTERNA,3 JUAN IRIBERRI,2 AND ISABEL BARCINA2

Departamento de Microbiología e Inmunología, Facultad de Farmacia, Apdo. 450, Universidad del País Vasco,
E-48080 Vitoria,1 and Facultad de Ciencias, Apdo. 644,2 and Facultad de Medicina, Apdo. 699,3
Universidad del País Vasco, E-48080 Bilbao, Spain

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The influence of biotic and abiotic factors on plasmid transfer between Escherichia coli strains in terms of the variation in the number of transconjugants formed and the variation in transfer frequency was investigated. The density of parent cells affected the number of transconjugants, reaching a maximum when the cell density was on the order of 109 CFU ml−1. As the donor-to-recipient ratios varied from 10−4 to 104, the number of transconjugants varied significantly (P < 0.001), reaching a maximum with donor-to-recipient ratios between 1 and 10. The concentration of total organic carbon in the mating medium affects both the number of transconjugants and the transfer frequency, being significantly higher (P < 0.001) when the total organic carbon concentration was higher than 1,139 mg of C liter−1. However, the transconjugants were detected even with less than 1 mg of C liter−1. Linear regression of log10 transconjugants versus mating temperature showed a highly significant regression line (P < 0.001). Neither the transfer frequency nor the transconjugant number varied significantly in the range of pHs assayed. We can conclude that plasmid transfer by conjugation can take place within a wide range of conditions, even in such adverse conditions as the absence of nutrients and low temperatures.

The significant increase in aquatic systems of the number of enterobacteria resistant to antibiotics is attributed to a combination of three factors: (i) the increase in dumping of wastewaters, (ii) a greater survival of some of these strains in aquatic systems, and (iii) resistance transfer processes. The indiscriminate use of antibiotics has given rise to an alarming increase in the number of resistant bacteria present in wastewater. The amount of wastewater dumped grows daily, both in urban and rural environments. The greater survival of these strains in aquatic systems may be due to the fact that in some cases, the resistance to antibiotics seems to be associated with resistance to environmental factors, such as light and metals (6, 7, 26). Numerous authors have demonstrated the transfer capacity of plasmids through conjugation, both in laboratory experiments (1, 10–12, 31, 38, 44) and in situ experiments using diverse microcosms (2, 17, 18, 29, 43).

The studies on plasmid transfer conjugation in aquatic systems have acquired greater relevance since the notable increase in the use of genetically engineered microorganisms in activities such as food and agricultural production, biocontrol of insects and diseases, metal and mineral leaching, environmental remediation, and wastewater treatment (3, 21, 27). We must not forget that the enterobacteria detected in aquatic systems, bearing plasmids of resistance to antibiotics and to other substances and factors, cannot be considered genetically engineered microorganisms in the strict sense of the term. However, like genetically engineered microorganisms, they do constitute a group of populations or bacterial strains with new genetic information, very different from that of the autochthonous bacterial population in aquatic systems; therefore, the same implications as for genetically engineered microorganisms in terms of ecological impact and health risks apply.

Once demonstrated, the possibility of transfer is essential to carry out studies on the influence of abiotic and biotic factors of the system in which the process of transfer frequency and transconjugant formation take place. In this way, the effects of temperature, pH, mating time, concentrations of nutrients and density of parent cells on plasmid transfer have been studied in epilithic bacteria (4–6, 16, 36, 37). However, biotic and abiotic factors affecting transfer in aquatic systems between free-living bacteria are rarely investigated (34).

The aim of this study is to examine how biotic and abiotic factors influence the transfer process between free-living bacteria. This influence will be evaluated according to the variation in the number of transconjugants formed and the frequency of transfer.

MATERIALS AND METHODS

Bacterial strains. An environmental Lac+, nalidixic acid-sensitive Escherichia coli strain containing natural plasmid bands and able to transfer some which confer resistance to ampicillin and gentamicin was used as the donor cell in all experiments. This E. coli strain was isolated in our laboratory from water from the Butron river (Vizcaya, Spain) and, after biochemical characterization, was examined for antimicrobial resistance by the MIC technique described by the National Committee for Clinical Laboratory Standards (33). The antimicrobial agent concentrations ranged from 1 to 128 μg ml−1. As the plasmidless recipient cell, we used a laboratory strain, E. coli K-12 strain 362 (Lac− F− Pro− His− Trp+); chromosomally resistant to nalidixic acid.

Culture media. Nutrient agar (Oxoid) supplemented with the appropriate antimicrobial drugs was used for maintenance of donor, recipient, and transconjugant strains at 4°C.

* Corresponding author.
Pure cultures were grown in Triptone Soya Broth (TSB; Oxoid) for susceptibility tests and conjugal transfer assays. MacConkey agar (Oxoid) was used to both select and enumerate donor, recipient, and transconjugant cells from mating assays.

Conjugal transfer assays. (i) Standard mating conditions. Separate 5-ml tubes of TSB were inoculated with an inoculum of overnight cultures of donor and recipient cells and incubated at 37°C with shaking to mid-log phase as determined by the optical density. Mating pairs were transferred to fresh medium to final densities of $2 \times 10^8$ and $1 \times 10^8$ CFU of donor and recipient cells ml$^{-1}$, respectively. Conjugal transfer was conducted at 20°C without shaking for a 2-h period.

To account for mutations of nalidixic acid resistance in the donor cells or of plasmid-encoded resistance to antibiotics in the recipient cells, we determined mutation frequencies by spreading each donor and recipient culture alone onto appropriate selective MacConkey agar plates (39). The frequencies obtained were always less than $10^{-7}$, lower than the sensitivity of our technique. To account for the possible role of transformation or viral transduction in mediating antibiotic resistance transfer, we mated the recipient strain with the cell-free supernatant from the donor culture in the same proportions; this was then spread onto the same selective agar plates for the selection of transconjugants. No transconjugants were collected in any of the culture media.

(ii) Modified mating conditions. Factors affecting transfer were tested in laboratory simulations. For this purpose, donor and recipient cells were grown to mid-log phase as described above, pelleted by centrifugation ($2,126 \times g$ for 15 min), and suspended to the original volume in the modified mating medium.

To assess the influence of Total Organic Carbon (TOC), mating pairs were suspended in ultrapure distilled water (MilliRo, MilliQ) (DW) and serially diluted in TSB in DW. The TOC concentration in the mating medium was measured with a Maihack Defor infrared analyzer (model Tocor 2; Westinghouse, Hamburg, Germany), and it ranged from 11.51 mg of C liter$^{-1}$ to less than 1 mg of C liter$^{-1}$.

In specific experiments carried out both in TSB and DW, the total cell density (donor and recipient) [D + R], donor-to-recipient ratio (D/R), pH, temperature, and mating time were varied at the beginning of each mating experiment.

Transfer frequency estimation. The frequency of transfer was estimated both as the number of transconjugants per initial number of donor cells (T/D) and as number of transconjugants per initial number of recipient cells (T/R).

Enumeration of bacteria. Before and after incubation, samples from matings were serially diluted in 0.85% NaCl solution and viable counts were done by a drop counting method, by using five 25-μl drops per dilution. Viable counts of donors and recipients were made on MacConkey agar plates supplemented, respectively, with nalidixic acid (50 μg ml$^{-1}$) to select recipient cells and ampicillin (128 μg ml$^{-1}$) to select donor cells. Transconjugants were selected and enumerated on MacConkey agar plates supplemented with both nalidixic acid (50 μg ml$^{-1}$) and ampicillin (128 μg ml$^{-1}$). Just before plating for the recovery of transconjugants, nalidixic acid (50 μg ml$^{-1}$, final concentration) was added to the mating medium to inhibit gene transfer. Colonies grown on MacConkey agar plates after a 24-h period at 37°C were recovered as CFU per milliliter.

Genetic characterization of recovered cells. To verify that the cells recovered on selective media were donor, recipient, or transconjugant cells, 10 or more colonies of each were picked and tested for their biochemical and antimicrobial characterizations and for the presence of plasmid bands. The presence of the plasmid bands in donor, recipient, and transconjugant cells was investigated by the alkaline procedure described by Birnboim and Doly (11a) and modified by Ish-Horowicz as described by Maniatis et al. (30) as well as by electrophoresis in a 0.7% agarose gel (ultrapure DNA grade; Bio-Rad Laboratories) as described by Meyers et al. (32).

Statistical analysis. Statistical tests were done with the StatView program developed for the MacIntosh computer. All counts and frequencies reported are the means of at least three determinations, and the coefficients of variation between replicate experiments were less than 12%. The differences between the means were detected by a one-way analysis of variance. Relationships between parameters measured were determined by regression analysis. Data that were heteroscedastic, as determined by measurements of the skew and kurtosis, were transformed to make them more homoscedastic, by using either log$_{10}(x + 1)$ or the arcsine square root. Probabilities less than or equal to 0.05 were considered significant (41).

RESULTS

Genetic characterization of recovered cells. The clear lysates from donor cells showed several DNA bands, and three of these bands were transferred by conjugation. In addition to these plasmid bands, the transconjugant cells also acquired resistance to ampicillin and gentamicin.

Influence of biotic factors on plasmid transfer. Figure 1 shows, with TSB as the mating medium, the variation in the number of transconjugants and the variation in transfer frequency depending on the variation of the initial cell density of the parent strains (D + R); in contrast, the D/R remains practically constant between 1 and 10. The number of transconjugants is seen to vary significantly ($p < 0.001$) as the cell density varies. The lowest number of transconjugants was detected when the cell density was on the order of $10^8$ CFU ml$^{-1}$, reaching a maximum when the cell density was on the order of $10^9$ CFU ml$^{-1}$. However, the transfer frequency, calculated as T/D or as T/R, remained practically constant throughout the range of cell densities studied.

By using ultrapure DW as the mating medium, transcon-
jugants were detected only with an initial density of parent cells of $10^7$ and $10^8$ CFU ml$^{-1}$. The maximum number of transconjugants, $5.8 \times 10^3$ CFU ml$^{-1}$, was obtained with a density of parent cells of $10^8$ CFU ml$^{-1}$; T/D gave values of $2.5 \times 10^{-4}$ and $3.1 \times 10^{-3}$, while T/R gave $4.7 \times 10^{-2}$ and $2.4 \times 10^{-1}$.

Figure 2A shows, with TSB as the mating medium, the variation in the number of transconjugants and the variation in the transfer frequency depending on the variation in the ratio of parent cells (D/R); in contrast, the cell density (D + R) remains constant at about $10^8$ CFU ml$^{-1}$. The number of transconjugants is seen to vary significantly ($P < 0.001$) as the D/R varies. Thus, the number of transconjugants detected increases as does the D/R, reaching a maximum value when the D/R is equal to 5.4; from here onwards, the number remains practically constant. As was the case with TSB as the mating medium, the dependence of the transfer frequency on the D/R differs clearly with the choice of one parameter or another (T/D or T/R). The transfer frequency values calculated as T/D do not show any significant variations within the D/R range studied, while those calculated as T/R clearly do.

### Influence of TOC concentration on plasmid transfer

The results corresponding to the influence of the concentration of TOC in the mating medium on the number of transconjugants obtained and on transfer frequencies are shown in Table 1. We observe a gradual decrease both in the number of transconjugants and in transfer frequencies as the concentration of TOC in the mating medium descends from 11.510 to less than 1 mg of C liter$^{-1}$, obtaining the maximum values with a TOC concentration of 11.510 mg of C liter$^{-1}$. The number of transconjugants obtained and the transfer frequency, calculated as T/D and T/R, respectively, remain constant when the TOC concentration in the mating medium varies from 1.139 to less than 1 mg of C liter$^{-1}$; the values in this range of TOC concentration are significantly lower ($P < 0.05$) than those obtained when the TOC concentration is 11.510 mg of C liter$^{-1}$.

#### Influence of pH on plasmid transfer

The pH values assayed are 6, 6.5, 7, 7.5, 8, and 8.5. When mating is carried out both in the absence of nutrients and in the TSB medium, the variation in pH gives rise to no significant differences either in the number of transconjugants obtained or in the transfer frequency values. In TSB, the number of transconjugants detected remains constant at about $10^6$ CFU ml$^{-1}$

### Table 1. Influence of TOC on plasmid transfer

<table>
<thead>
<tr>
<th>TOC (mg of C/liter)</th>
<th>Mean no. of transconjugants (CFU/ml) ± SE</th>
<th>Mean transfer frequency ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.510</td>
<td>(7.41 ± 0.41) x 10^{-3} (2.06 ± 0.10) x 10^{-2}</td>
<td>T/D</td>
</tr>
<tr>
<td>1.139</td>
<td>(8.41 ± 0.40) x 10^{-3} (2.70 ± 0.15) x 10^{-2}</td>
<td>T/R</td>
</tr>
<tr>
<td>119</td>
<td>(6.76 ± 0.21) x 10^{-4} (1.77 ± 0.07) x 10^{-3}</td>
<td>T/D</td>
</tr>
<tr>
<td>11</td>
<td>(7.29 ± 0.42) x 10^{-4} (3.85 ± 0.20) x 10^{-3}</td>
<td>T/R</td>
</tr>
<tr>
<td>1</td>
<td>(1.96 ± 0.07) x 10^{-4} (6.76 ± 0.30) x 10^{-4}</td>
<td>T/D</td>
</tr>
<tr>
<td>&lt;1</td>
<td>(1.00 ± 0.06) x 10^{-4} (3.61 ± 0.17) x 10^{-4}</td>
<td>T/R</td>
</tr>
<tr>
<td>1&lt;1</td>
<td>(8.00 ± 0.39) x 10^{-5} (2.89 ± 0.14) x 10^{-4}</td>
<td>T/D</td>
</tr>
</tbody>
</table>

* DW.
and the transfer frequency values are on the order of $10^{-3}$, calculated as T/D, or on the order of $10^{-2}$, calculated as T/R. When mating is carried out in ultrapure DW, the number of transconjugants is on the order of $10^2$ CFU ml$^{-1}$ and the transfer frequency values remain about $10^{-5}$, calculated as either T/D or T/R.

**Influence of mating temperature on plasmid transfer.** The results corresponding to the effect of mating temperature on transfer frequency and on the number of transconjugants obtained using TSB as the mating medium are shown in Fig. 3A. The range of temperature assayed is from 8 to 37°C. The number of transconjugants is observed to vary significantly ($P < 0.001$) with the temperature. The number of transconjugants remains constant at 8 and 15°C; from 15°C upwards, it increases progressively, reaching its maximum value at 37°C. A linear regression analysis of the log$_{10}$ transconjugant number versus temperature shows a highly significant regression line ($P < 0.001$). The transfer frequency varies significantly with the temperature, both when calculated as T/D ($P < 0.001$) and as T/R ($P < 0.01$); in both cases, it presents a plot similar to that described for the transconjugant number. Moreover, a linear regression analysis of log$_{10}$ transfer frequencies versus temperature also shows a significant regression line ($P < 0.01$ for T/D and $P < 0.05$ for T/R).

We must point out that the slopes for the three regression lines obtained show the same value, 0.13. This slope indicates that a 7.7°C change in temperature led to a 10-fold change in the transconjugant number and in the transfer frequency.

When mating is carried out in the absence of nutrients, the results are clearly different (see Fig. 3B). In the temperature range tested, we observed no significant differences in either the number of transconjugants obtained or the transfer frequency values for either T/D or T/R.

**Influence of mating time on plasmid transfer.** Mating assays were carried out with two different incubation times, 1 and 2 h, by using TSB and ultrapure DW as the mating media. By using TSB as the mating medium, the number of transconjugants, $2.04 \times 10^6$ CFU ml$^{-1}$, obtained after 2 h of mating time is higher, though not significantly so, than that obtained after 1 h, $5.6 \times 10^5$ CFU ml$^{-1}$. The transfer frequency after 2 h is higher than that detected after 1 h, for both T/D and T/R. Transfer frequency values at 1 and 2 h were $1.21 \times 10^{-3}$ and $5.7 \times 10^{-3}$, respectively, for T/D and $1.69 \times 10^{-3}$ and $6.76 \times 10^{-3}$, respectively, for T/R.

By using ultrapure DW as the mating medium, we observe no significant differences in either the number of transconjugants obtained, $2.0 \times 10^4$ CFU ml$^{-1}$ at 1 h and $1.2 \times 10^4$ CFU ml$^{-1}$ at 2 h, or the frequency values, whether calculated as T/D ($3.3 \times 10^{-3}$ at 1 h and $1.4 \times 10^{-5}$ at 2 h) or as T/R ($1.2 \times 10^{-3}$ at 1 h and $4.9 \times 10^{-4}$ at 2 h).

We must also point out that there is no significant increase in the number of parent cells during the mating times used.

**DISCUSSION**

The results shown in Fig. 1 show a direct relationship between the initial density of parent cell (D + R) and the number of transconjugants detected ($r = 0.985; P < 0.001$), and therefore the transfer frequency does not vary throughout the range of cell densities studied. Since the aim of this study is to examine the influence of various factors on gene transfer and bearing in mind that transfer frequency is always calculated as the number of transconjugants obtained, throughout our research we use initial parent cell density of $10^8$ CFU ml$^{-1}$, which enables us to detect the greatest number of transconjugants. A relatively high transconjugant number allows us to evaluate, through the measurable variations in that number, the possible effects of the factors studied on gene transfer. If, on the other hand, the number of transconjugants detected is low, any negative effect on gene transfer could provoke a drop in that number to nondetectable levels; this could then be erroneously interpreted as an inhibition of gene transfer. Thus, Fry and Day (14) report that it is very important to avoid using very low initial densities of cells (less than $2 \times 10^3$ bacteria cm$^{-2}$) or too few donors, as plasmid frequencies drop rapidly below optimum numbers. Sandt and Herson (38) report that high densities of parent organisms (on the order of $10^8$ to $10^9$ CFU ml$^{-1}$) are required to obtain triparental recombinants in drinking water. With respect to the relationships between the D/R and the number of transconjugants and bearing in mind the aforementioned considerations, throughout our research we use a D/R of between 1 and 10, as this is the value which enables us to obtain the maximum number of transconjugants (Fig. 2).

As we have shown in Results, the two parameters used to calculate transfer frequency vary according to the D/R. When we use TSB as the mating medium, both parameters remain constant within determined ranges of variation of D/R (Fig. 2A). Thus, T/D remains constant when the density of recipient cells is higher than that of donor cells because
there is a direct relationship between the transconjugant number and the donor cell density (r = 0.984; P < 0.01). On the other hand, T/R remains constant when the donor cell density is in excess because then the transconjugant number is directly related to the recipient cell density (r = 0.975; P < 0.01). By using ultrapure DW as the mating medium (Fig. 2B), T/D remains constant throughout the entire range of variation of D/R, while T/R is directly related (r = 0.826; P < 0.05) to the D/R. These increases in T/R do not, however, correspond to a real increase in transfer frequency; they reflect a decrease in the density of recipient cells, as the number of transconjugants does not vary as the D/R increases. The maximum transfer frequency values, calculated as T/D and T/R, are obtained within the range of variation of D/R in which both parameters remain constant (Fig. 2A). This D/R variation range, moreover, includes the D/Rs mentioned by other authors as ideal to obtain maximum transfer frequency values. Thus, Gauthier et al. (16) report maximum frequencies for the D/R of 1/1, Bale et al. (6) also find that the T/Rs are related to the D/Rs and that high ratios favor transfer, and Rochelle et al. (37) detect maximum frequency values (T/R) when the D/R oscillates between 0.4 and 30.

An analysis and interpretation of the results obtained in experimental studies normally includes a comparison with the results obtained by other authors. Very often, the results referring to one particular phenomenon are obtained through very different methodologies; this makes it very difficult to compare data, and it is necessary to assume the differences implicit in this disparity. An added difficulty exists in the case of studies on gene transfer because the object under study, the transfer frequency, is not calculated in the same way by all authors. In studies on gene transfer in aquatic systems, Shw and Cabelli (39), Mach and Grimes (29), Corliss et al. (12), Altherr and Kasweck (2), Trevors and Oddie (44), Cruz-Cruz et al. (13), and O’Morchoe et al. (34) calculate the frequency as number of transconjugants per initial number of donor cells, while Bale et al. (4–6), Rochelle et al. (36, 37), Fry and Day (14), and Gauthier and Brettmayer (15) calculate it as number of transconjugants per initial number of recipient cells. Moreover, other authors, such as Richaume et al. (35), substitute the initial number of donor or recipient cells for the final number of surviving cells of the long periods of incubation used in their mating assays. None of this would be particularly important if all the methods of calculation gave similar frequency values for the same experiment. The truth is, however, that the results obtained throughout this study allow us to deduce that the use of one formula or another in the calculation of transfer frequency will give clearly different results, except when the D/R is equal to 1. We, therefore, believe that, in order to study the influence of abiotic factors on transfer frequency, the results must be expressed as T/D or T/R in relation to D/R and to the mating medium used in the mating assay in such a way that experimental variations in the D/R have no influence on transfer frequency values. Any variations detected can then be attributed exclusively to the effect of the abiotic factors whose influence we wish to determine.

The results obtained in experiments conducted to determine the effect of the concentration of TOC on transfer indicate that plasmid transfer is clearly influenced by the availability of carbon (see Table 1). Other authors also find a close link between the transfer and availability of nutrients where frequency increases along with the nutrient concentration in the medium. Thus, Trevors and Oddie (44) found that plasmid transfer in sterile stream water occurs only when it is amended with dilute nutrient broth (0.8 ml of nutrient broth per water sample). Sandt and Herson (38) found that relatively large increases in the levels of nutrients in the environment increase the frequency of plasmid mobilization, which varied from 5.2 × 10⁻³ in drinking water to 5.9 × 10⁻³ in TSB medium; however, the results of matings in water supplemented with TSB (15 vol/vol, in water) were not significantly different from those of matings in water. TOC concentrations were 1.5 to 7 mg of C liter⁻¹ in water and 20 mg of C liter⁻¹ in 1% TSB. Our results (see Table 1) coincide with those of Trevors and Oddie in that, in our case, the transfer frequency does not vary within the range of TOC concentration between 1,139 and <1 mg of C liter⁻¹.

None of these authors, however, uses TOC concentrations with such a wide range or in such low concentrations as those tested by us, from less than 1 to 11,530 mg of C liter⁻¹. Rochelle et al. (37) detect transfer frequencies as T/R from 4.8 × 10⁻⁷ to 5.7 × 10⁻³ in a range of carbon concentrations from 0 to over 10 g of C liter⁻¹. However, we must take into account that these authors calculate carbon concentrations on the basis of the chemical composition of the nutrients used and that in the case of the minimum carbon concentration, 0 g of C liter⁻¹, mating is carried out on Bacto Agar (0140-01; Difco) with no added carbon in the form of Standard Plate Count Agar (Oxoid CM 463). The authors themselves point out that the values do not take into account any available carbon that may have been present in the agar. Moreover, our results for the influence of carbon concentration on transfer are not really comparable to those of these other authors, as they work with attached bacteria, epilithes, while we work with free-living bacteria; the carbon incorporation of these two types of bacteria, attached and free-living, is clearly different (22–24). In this sense, we agree with Rochelle et al. (37), who report that the increase of transfer frequencies with nutrient concentration indicates that the physiology of the bacteria involved in conjugation is an important determinant.

The results obtained in these experiments also indicate that the plasmid transfer takes place even when there is practically no carbon available, less than 1 mg of C liter⁻¹ (see Table 1). The relatively high transfer frequency detected in ultrapure DW (T/D equal to 8 × 10⁻³ and T/R equal to 2.9 × 10⁻⁴) is probably due to the fact that the physiological state of the E. coli cells does not undergo significant variations, since the mating time (2 h) used in our mating assays is short. We base these considerations on results obtained by Barcina et al. (8, 9) in previous studies on the variations of the physiological state of E. coli cells throughout a process of dormancy as a survival strategy in aquatic systems. We, therefore, believe that a scarcity of nutrients in natural systems does not constitute an absolute impediment to gene transfer in these systems, although the transfer frequency may indeed decrease.

Variations in pH within a range from 6 to 8.5 do not significantly affect the frequency or the number of transconjugants detected, both when TSB is the mating medium and when mating is carried out in the absence of nutrients. Other authors such as Stotzky and Kasrovsy (42), Kasrovsy and Stotzky (28), and Richaume et al. (35) working on soils find maximum transfer frequencies at pH values close to neutral, while acid pH values affect the process negatively. Rochelle et al. (37) report that there is no effect on transfer within a pH range from 5.0 to 8.0 at 25°C.

Numerous authors, such as Grabow et al. (19), Singleton and Anson (40), Mach and Grimes (29), Altherr and Kasweck (2), Gauthier et al. (16), Cruz-Cruz et al. (13), Bale
et al. (4, 5), O’Morchoe et al. (34), Richaume et al. (35), Rochelle et al. (37), and Sandt and Herson (38), have studied the effect of temperature on transfer. As a rule, they find that low temperatures have a negative effect on transfer, detecting maximum frequencies in the range of 20 to 30°C. Our results (Fig. 3) also indicate a negative effect of temperature on transfer, as the frequencies detected at low temperatures (8 and 15°C) are significantly lower than those detected in the higher temperatures assayed (20, 28, and 37°C). We, therefore, agree with Rochelle et al. (36), who report that conjugation will be more likely to take place at temperatures higher than would normally be expected in temperate aquatic environments.

Unlike the results found by other authors, who report a minimum temperature of higher than 15°C for conjugal transfer involving R plasmids in E. coli (40, 44, 45), we detected transconjugant formation at a mating temperature of 8°C, even in the absence of nutrients (Fig. 3). From these results, we can deduce that both low temperatures and the joint action of low temperatures and absence of nutrients do not totally inhibit plasmid transfer.

Transfer frequency has an exponential relationship to mating temperature within the 8 to 37°C range. Other authors (6, 14) also find that the frequency log_{10} is linearly related to temperature between 6 and 21°C. This relationship shows that temperature is an important factor in transfer assays, and this could explain to a large extent the variation in transfer frequencies (R^2 = 93.7% for T/D in this work). All of this is logical if we bear in mind that conjugation is an active process and that heterotrophic bacterial activity, measured through the rate of incorporation of isotopically radiolabeled substrates (25), has an exponential relationship to temperature. This hypothesis is confirmed by the results obtained in this study on the effect of temperature on transfer in the absence of nutrients in the mating medium. Thus, given that there is no possibility in this case of incorporating nutrients, no significant variations are observed in the frequency in response to the increase in temperature.

With respect to the incubation times used in the mating assays, Rochelle et al. (37) detect maximum frequencies after 5 h of incubation, when transfer takes place between parent cultures, and after 24 h, when it is carried out from the mixed natural suspensions of epithelial bacteria to Pseudomonas aeruginosa. Graves and Riggs (20), Gauthier et al. (16), and Rochelle et al. (36) also detect maximum transfer frequencies after 24 h of mating time. In our case, on the basis of results obtained from the experiments carried out to observe the influence of mating time on transfer, we fixed the mating time at 2 h. No growth in the parent cell population is observed in this time, and it also permits us to obtain a high enough number of transconjugants to be able to evaluate the effect of various factors through the variations in that number. While this mating time is notably shorter than that used by the aforementioned authors, we believe that prolonged periods of incubation can lead to a nonhomogeneous growth in the number of parent cells and transconjugants. In this case, the use of the initial cell density in the calculations of T/D and T/R could therefore lead to an overestimation of the transfer frequency.

From the results obtained, we can conclude that plasmid transfer by conjugation can take place within a wide range of conditions, even in such adverse conditions as absence of nutrients and low temperatures. This indicates that in general, the abiotic conditions of natural aquatic systems do not represent an insurmountable barrier for plasmid transfer to take place. On the other hand, the limited response of frequency, in the absence of nutrients, to the variations in the different parameters studied indicates that the availability of nutrients may be considered to be one of the factors of greatest influence on frequency.

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