APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1994, p. 4273–4278
0099-2240/94/$04.00+0

Vol. 60, No. 12

Effect of Growth Phase and Parental Cell Survival in River Water on Plasmid Transfer between *Escherichia coli* Strains

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Received 12 May 1994/Accepted 15 September 1994

We evaluated the transfer to and from *Escherichia coli* of endogenously isolated plasmid material from the River Butrón during the growth of three donor strains and two recipient strains as well as after the survival of these parental cells in river water. Transfer frequency varied greatly during the growth of donor cells, with minimum values in the exponential phase; frequency remained constant, however, during the growth of recipient strains. After survival in river water, donor cells lost their ability for plasmid transfer before any other physiological variations in the cells caused by environmental stress were detected. Under the same conditions and during equal periods, however, no variation in the ability of recipient cells to receive and express plasmid material was observed.

Various reasons of sanitary interest and ecological interest highlight the importance of studying and quantifying plasmid transfer processes in aquatic systems. First, many authors have demonstrated the transfer capacity of natural plasmids through conjugation in both laboratory (14, 35, 36) and in situ experiments (1, 4, 19, 21, 32) with diverse microcosms. Second, in aquatic systems there has been an increase in the number of bacteria that harbor R plasmids (8, 28) and thus may be considered potential donors. A third reason is 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 (2, 22, 26), all of which involve the risk of accidental release.

Transfer frequencies calculated in aquatic systems are generally lower than those observed in a laboratory or microcosms (25, 32, 36). However, we know very little about the mechanisms which promote or impede naturally occurring plasmid transfers in aquatic systems. Not only is this a very complex phenomenon indeed, but it is also very difficult to study, mainly because of the great number of factors which influence the results. Some works (3, 14, 18, 33, 35) in which the influence of total organic carbon, pH, temperature, coincubation time, and the density and ratio of parental cells in mating media on transfer processes have been studied do exist. Freter (16) points out that fertility depends not only on the nature of the plasmids and interacting bacteria but also to a large degree on the physiological state of the bacteria. However, this aspect has not been extensively studied in aquatic systems.

In light of the above, when evaluating the importance of transfer processes in aquatic systems in which the potential donor and recipient cells are enterobacteria, we must also consider the survival strategies of parental cells and transconjugants to be of considerable importance. If we consider the physiological variations undergone by *Escherichia coli* strains as a response to environmental stress (5, 6, 7), it is conceivable that such changes affect the ability of cells to act as donors and recipients in transfer processes. Recently, Goodman et al. (20) demonstrated that prestarvation of an *E. coli* strain affects its ability to transfer plasmids.

The aim of this work was to determine whether the growth phases of donors and recipients could affect conjugal plasmid transfer and whether parental strains could retain their functions as donors or recipients after survival in river water under nonilluminated and illuminated conditions.

MATERIALS AND METHODS

Bacterial strains. Five *E. coli* strains were used in this study. The most relevant characteristics of these strains are listed in Table 1. EC_{15} is an environmental strain isolated in our laboratory from River Butrón water. This strain contains natural plasmid bands and is able to transfer some which confer resistance to ampicillin and gentamicin. Plasmid-free strains 416S, a spontaneous streptomycin-resistant mutant of strain STCC 416 (ATCC 27325), and J62, a K-12 strain, are the wild-type parents of plasmid-bearing strains $416S_{15}$ and $J62_{15}$, respectively. Strains were examined for antimicrobial agent resistance by the MIC technique (27). Antimicrobial agent concentrations ranged from 1 to 128 $\mu g\ ml^{-1}$. The presence of plasmid bands in donor, recipient, and transconjugant cells was investigated by the alkaline procedure and electrophoresis on a 0.7% agarose gel (ultrapure DNA grade; Bio-Rad) as described by Sambrook et al. (34). Organisms were maintained at 4°C on nutrient agar (NA; Oxoid) supplemented with appropriate antimicrobial drugs.

River water samples. All river water samples were collected from River Butrón, a slow, short stream on the northern coast of Spain. The river has a surface area of 175.15 km^2. River Butrón runs through a basically agricultural area, its mean flow rate is 3 $m^3\ s^{-1}$, and it receives sewage from several municipalities. Samples were collected at a station at 42°22′N, 2°51′W between 8:00 and 10:00 a.m. from a depth of approximately 0.5 m and at a distance of 3 m from the riverbank in sterilized bottles precleaned with diluted HCl. All experiments were initiated within 2 h of sampling.

Direct bacterial counts. Total number of cells was enumerated directly by epifluorescence microscopy by using the

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TABLE 1. *E. coli* strains used in this study

Strain	Source	Relevant phenotype[a]	Plasmid bands
EC_{15}	Environment	Lac^+ Am^r Gm^r Nal^s Str^s	Yes
416S	Streptomycin-resistant mutant of STCC 416 (ATCC 27325)	Lac^+ prototrophic Am^s Gm^s Nal^s Str^r	No
J62	Laboratory K-12 strain	Lac^- Pro^- His^- Trp^- Am^s Gm^s Nal^r Str^s	No
$416S_{15}$	Derived by us from EC_{15} × 416S matings	Lac^+ prototrophic Am^r Gm^r Nal^s Str^r	Yes
$J62_{15}$	Derived by us from EC_{15} × J62 matings	Lac^- Pro^- His^- Trp^- Am^r Gm^r Nal^r Str^s	Yes

[a] Abbreviations: Am, ampicillin; Gm, gentamicin; Nal, nalidixic acid; Str, streptomycin; Lac, lactose; Pro, proline; His, histidine; Trp, tryptophan; s, sensitive; r, resistant.

standard acridine orange direct count procedure of Hobbie et al. (24).

Bacterial size measurements. The lengths of at least 200 bacteria were measured from acridine orange-stained subsamples by image analysis. This equipment includes a Nikon epifluorescence microscope integrated with a video camera (Hamamatsu C2400) and a semiautomatic image analysis system (VIDS IV; 286 modular B10S V3.03 NFS).

Heterotrophic bacterial activity. Heterotrophic bacterial activity was estimated from glucose uptake rates. Three subsamples were analyzed for each uptake measurement. The subsample volume was 5 ml, and the volume of each incubation flask was 50 ml. Subsamples were incubated with [U-^{14}C]glucose (230 to 350 mCi mmol^{-1}; Amersham) at a final concentration of 250 μg liter^{-1} and cold glucose (Merck) at final concentrations of 249.75 and 12.25 mg liter^{-1} for EC_{15} and J62, respectively. These concentrations were chosen as a result of previous studies in which we used the kinetic approach method of Wright and Hobbie (40, 41). Subsamples were incubated for 30 min in the dark at 20°C with shaking. After this incubation period, 40 μl of H_2SO_4 (2 N) was injected into the flasks, and samples were further incubated for 1 h to trap the $^{14}CO_2$ released with a filter paper impregnated with β-phenylethylamine (23). After both incubation periods, the entire volume of each sample was filtered through 0.2-μm-pore-size cellulose acetate filters (Sartorious). Filters were rinsed three times with 5 ml of filtered water (0.2-μm-pore-size membrane filters), placed in 10 ml of scintillation fluid (Cocktail F-1 Normascint; Scharlau), and radioassayed by liquid scintillation counting. Quench curves were computed by the channel ratio method. Controls for abiotic absorption were prepared in a similar manner, except that 2% formaldehyde (final concentration) was added. Assimilated and respired fractions were defined as described by Wright and Burnison (39).

Conjugal transfer assays. *E. coli* EC_{15}, $416S_{15}$, and $J62_{15}$ were used as donors, and *E. coli* 416S and J62 were used as plasmidless recipients. Conjugal transfer assays were mainly performed as described by Fernandez-Astorga et al. (14). Mating pairs were suspended in tryptone soy broth (TSB; Oxoid) to a final density of about 10^8 CFU ml^{-1} and with a donor-to-recipient ratio of 1/10. Conjugal transfer was conducted at 20°C without shaking for 2 h.

CFU counts of parental strains were made on MacConkey agar plates (Oxoid) supplemented with gentamicin (32 μg ml^{-1}) for donor cells, with streptomycin (100 μg ml^{-1}) for 416S recipient cells, and with nalidixic acid (50 μg ml^{-1}) for J62 recipient cells. Transconjugants were selected and enumerated on MacConkey agar supplemented with the appropriate antimicrobial drugs at the above concentrations. In order to estimate plate matings, each pair of donors and recipients was mixed and immediately plated on transconjugant-selective plates. These plates showed no recovery of transconjugants. The transfer frequency was estimated as the number of transconjugants per initial number of donor cells.

Growth curves. The ability for conjugal plasmid transfer of donor and recipient cells throughout growth curves was evaluated by using batch cultures of parental strains. Separate flasks with TSB medium were inoculated with overnight cultures of each strain and incubated at 37°C with shaking at 160 rpm. Aliquots were collected for direct bacterial counts, CFU counts, bacterial size measurements, and conjugal transfer assays. CFU counts were performed on MacConkey agar plates supplemented with appropriate antimicrobial drugs. Conjugal transfer assays were mainly conducted as described above, but in every mating, the cells of one parental strain were harvested at different growth phases and the cells of the other one were harvested at early stationary phase.

Survival experiments. To evaluate the ability for conjugal plasmid transfer throughout the survival of donor and recipient cells, suspensions of parental strains maintained in River Butrón water were used. Cultures were grown in TSB for 18 h at 37°C with shaking at 160 rpm. Cells were harvested by centrifugation (3,000 × *g* for 15 min) and washed three times in sterile saline solution (0.85% NaCl solution). Flasks with sterile river water filtered through 0.2-μm-pore-size membrane filters (Millipore) were inoculated with cells at a final density of 10^8 cells ml^{-1}. Inoculated flasks were incubated for 96 h at 20°C with shaking at 160 rpm in an orbital incubator. In illuminated experiments, flasks were incubated with an illumination system consisting of eight Sylvania CW-ST 133 18-W lamps (5, 6). Aliquots were collected for direct bacterial counts, CFU counts, bacterial size measurements, heterotrophic bacterial activity estimations, and conjugal transfer assays. CFU counts were performed on NA and MacConkey agar plates supplemented with appropriate antimicrobial drugs. Conjugal transfer assays were done by the procedure described above, but in every mating, the cells of one parental strain were assayed at different survival stages and those of the other one were assayed at early stationary phase.

Statistical analysis. Statistical tests were done with the StatView program (Apple 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 means were detected by one-way analysis of variance. Probabilities of ≤ 0.05 were considered significant.

RESULTS

Figure 1 shows the transfer frequency values during the growth cycles in a batch culture of TSB of donor strains EC_{15}, $416S_{15}$, and $J62_{15}$ with recipient cells in the early-stationary-growth phase. In all three cases, transfer frequencies varied significantly along the growth curves of donor cells. The minimum transfer frequency values were observed in the

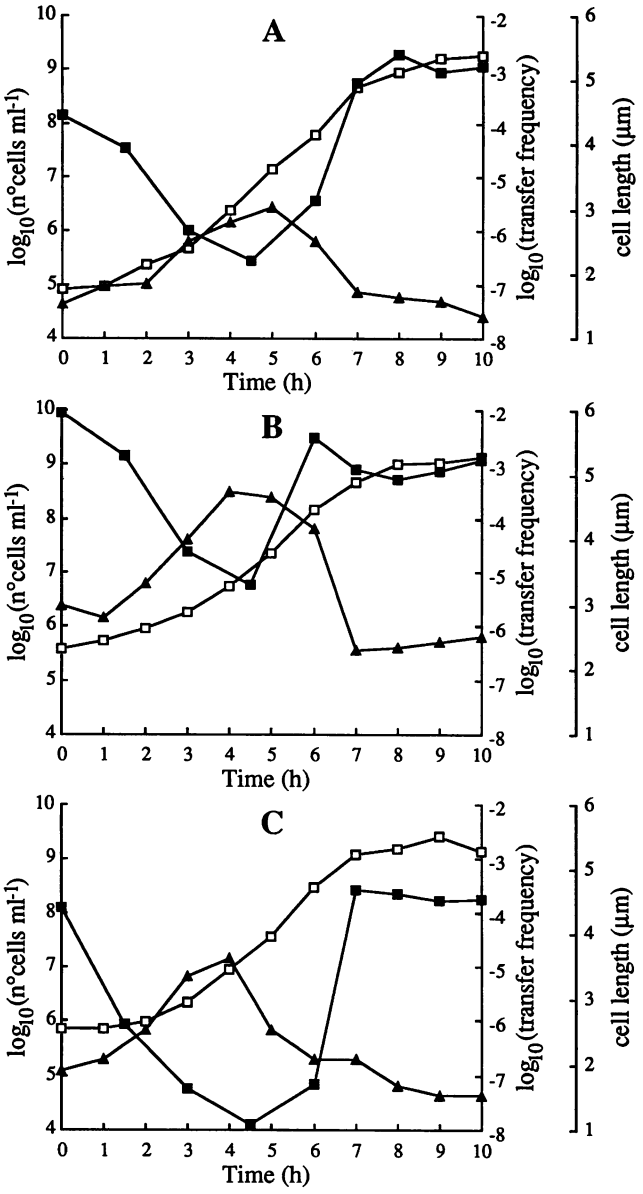

FIG. 1. Changes in transfer frequency (■), cell length (▲), and total number of donor cells (□) during the growth curves of donor strains EC_{15} (A), $416S_{15}$ (B), and $J62_{15}$ (C) in batch cultures of TSB. Strain J62 was used as the recipient in matings with EC_{15} and $416S_{15}$. Strain 416S was used as the recipient in matings with $J62_{15}$.

exponential-growth phase (2.86×10^{-7}, 5.97×10^{-6}, and 1.30×10^{-8}, respectively). We must point out that the mean size of donor cells also varied significantly along the growth curve (Fig. 1). In all three cases, the change in cell size was the opposite of that detected for transfer frequency. Thus, the maximum cell sizes (3.03, 4.74, and 3.62 μm, respectively), which were detected in the middle of the exponential-growth phase, correspond to the minimum values of transfer frequency (Fig. 1).

Figure 2 shows the transfer frequency values during the growth cycles of recipient strains 416S and J62 in a batch culture of TSB with donor cells in early-stationary-growth phase. In these experiments, we detected no significant differences in transfer frequency along the growth curves of these

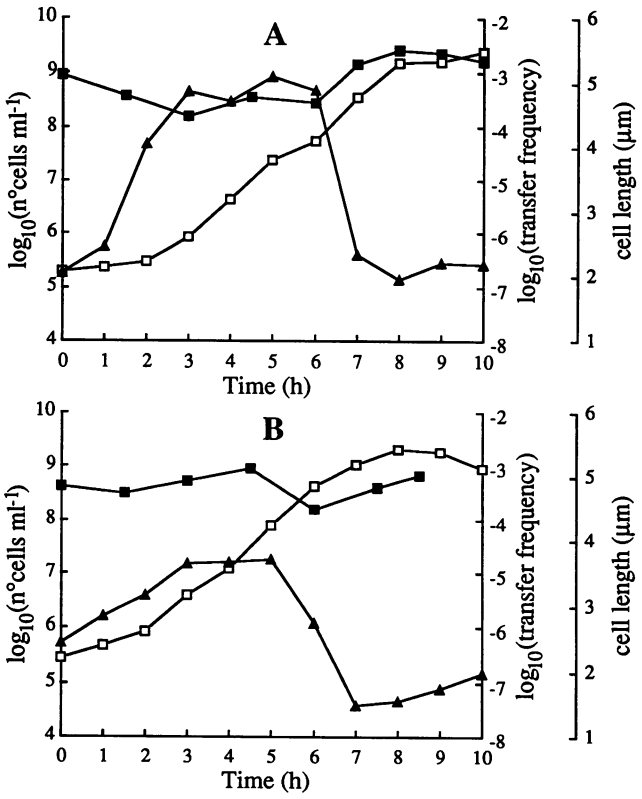

FIG. 2. Changes in transfer frequency (■), cell length (▲), and total number of recipient cells (□) during the growth curves of recipient strains 416S (A) and J62 (B) in batch cultures of TSB. Strain EC_{15} was used as the donor in both cases.

two recipient strains. The mean size of recipient cells varied significantly along each growth curve (Fig. 2). The largest cell sizes (3.7 and 5.1 μm, respectively) in this case were also detected in the exponential-growth phase.

Figures 3 and 4 show the results obtained during long-term survival assays of donor strain EC_{15} in River Butrón water. In nonilluminated systems (Fig. 3A and B), the cell size, total number of cells, number of culturable cells, glucose uptake rate, and percentages of glucose assimilated and respired remained constant throughout incubation. However, the number of transconjugants formed in the two matings (EC_{15} × 416S and EC_{15} × J62) decreased after 8 and 4 h, respectively; in both cases, no transconjugants were detected after the donor strain, EC_{15}, had remained in river water for longer than 24 h. However, there was an apparent maintenance of plasmids, because CFU counts on MacConkey agar with gentamicin remained constant throughout experimentation. The results obtained with the other two donor strains, $416S_{15}$ and $J62_{15}$, in nonilluminated river water (data not shown) were similar to those with strain EC_{15}.

In illuminated microcosms (Fig. 4), only the cell size and total number of cells of strain EC_{15} remained constant. After 12 h, the glucose uptake rate decreased and the percentages of glucose assimilated and respired changed, with the former falling and the latter rising (Fig. 4B). The number of culturable cells also decreased after 48 h of incubation. Under these conditions, the number of transconjugants formed in the two matings (EC_{15} × 416S and EC_{15} × J62) decreased from the beginning of the experiment, and no transconjugants were

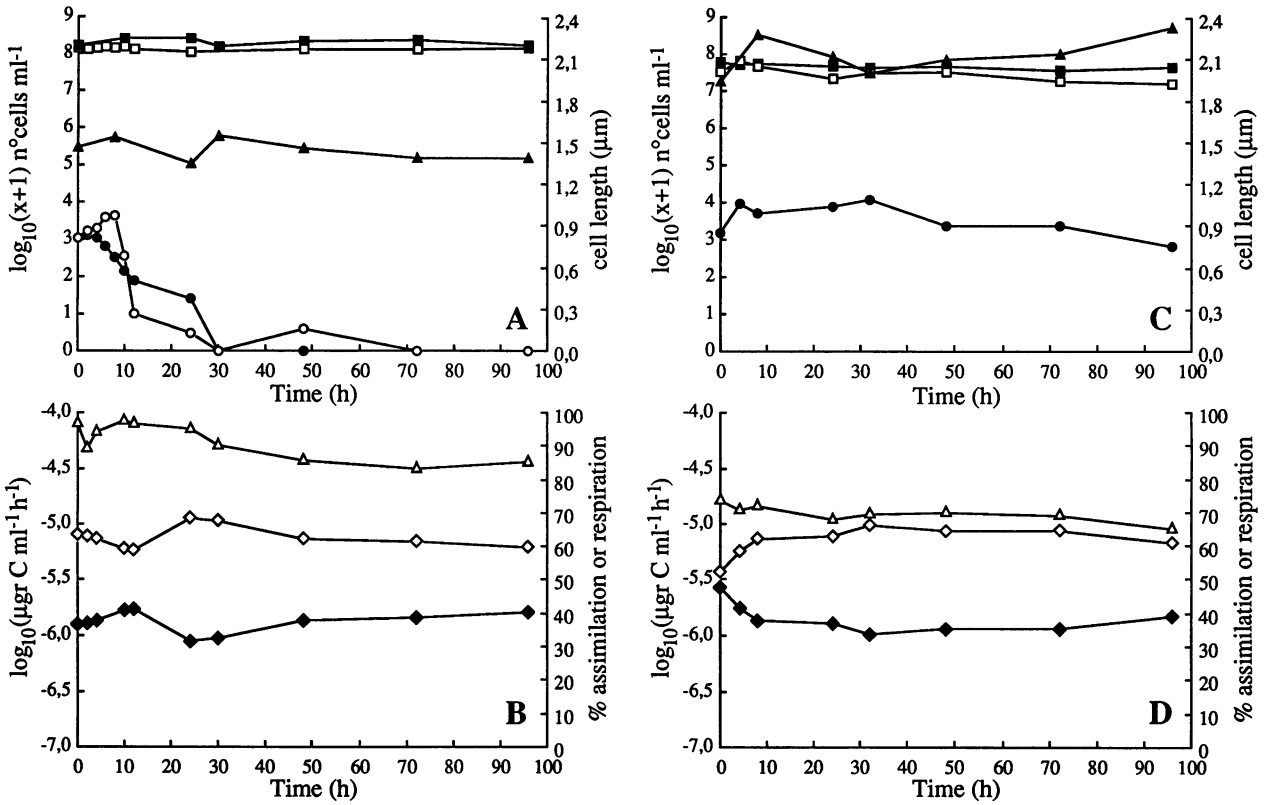

FIG. 3. Changes in bacterial counts and heterotrophic bacterial activities during long-term survival in sterile river water of donor strain EC_{15} (A and B) and recipient strain J62 (C and D) in nonilluminated systems. (A and C) ■, total number of cells; □, CFU on NA; ▲, cell length. For transconjugants, CFU rendered in matings with EC_{15} as donor cells and with 416S (○) or J62 (●) as recipient cells are indicated. The lowest detectable cell density was 3 CFU ml^{-1}. (B and D) △, total glucose uptake rate; ◇, percentage of assimilation; ◆, percentage of respiration.

detected after the donor strain had remained in illuminated river water for 8 h or 24 h, respectively (Fig. 4A). However, at that time, there was an apparent maintenance of plasmids, because CFU counts on MacConkey agar with gentamicin remained constant for 48 h of incubation.

Figure 3C shows the changes in cell size, total number of cells, and number of CFU of recipient strain J62 in the microcosm of water from the River Butrón under nonilluminated conditions as well as the number of transconjugants obtained with EC_{15} as the donor. In Fig. 3D, we can observe the glucose uptake rate for J62 as well as the percentages of glucose assimilated and respired by this strain. Under these conditions, all of the parameters quantified, including the number of transconjugants formed in EC_{15} × J62 matings, remained constant during the survival of the recipient strain in river water microcosms. Similar results were obtained for the survival process of recipient strain 416S in nonilluminated river water (data not shown).

DISCUSSION

Since conjugation is an active process (14, 16, 30, 38), the physiological states of donor and recipient cells strongly influence the transfer process. According to our results, the respective influences of the physiological states of donor and recipient cells on the results of the transfer process are clearly different. Thus, the number of transconjugants formed is greatly influenced by the physiological state of the donor. However, the physiological state of recipient cells does not seem to influence the number of transconjugants rendered.

As we have seen (Fig. 1), transfer frequency varied dramatically along the growth curves of the three donor strains used in this study (EC_{15}, $416S_{15}$, and $J62_{15}$). On the other hand, no variations in transfer frequency were detected along the growth curves of the recipient strains, 416S and J62 (Fig. 2).

In the case of donor cells, the greatest variations in frequency took place in the exponential phase. According to Walmsley (37), under normal physiological conditions, the limiting step in pair formation is the production of F pili by donors. Novotny and Lavin (29) observed that maximum piliation is attained at the end of the exponential phase. Coincidentally, in this work, high transfer frequency was detected in the later stages of exponential-phase growth.

According to these results, and as a working hypothesis, plasmid transfer could be considered a survival strategy of genetic information. During exponential growth, the survival of information is assured by the generation of new cells, which means that the mechanisms of genetic transfer are minimized. In addition, during exponential growth, significant variations in cell size are detected. The largest cells detected in the exponential phase transfer plasmids with minimum efficiency (Fig. 1). All the energy in these cells may be channeled into processes of biosynthesis to the detriment of transfer processes. On the other hand, genetic transfer becomes relevant under conditions unfavorable for population growth. In batch cultures, according to Brown et al. (9), these conditions might

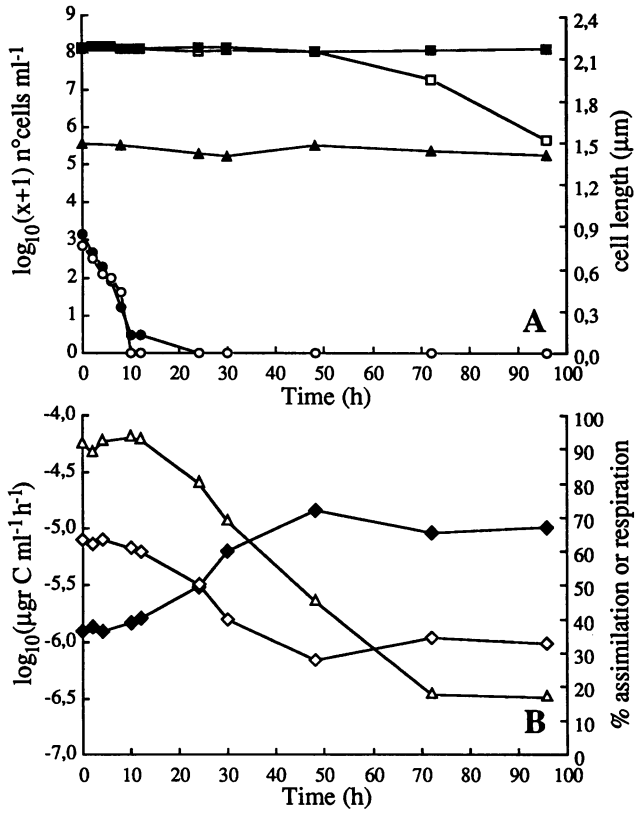

FIG. 4. Changes in bacterial counts and heterotrophic bacterial activities during long-term survival in sterile river water of donor strain EC_{15} in illuminated systems. (A) ■, total number of cells; □, CFU on NA; ▲, cell length. For transconjugants, CFU rendered in matings with EC_{15} as donor cells and with 416S (○) or J62 (●) as recipient cells are indicated. The lowest detectable cell density was 3 CFU ml^{-1}. (B) △, total glucose uptake rate; ◇, percentage of assimilation; ◆, percentage of respiration.

arise several generations before the onset of the stationary phase because of a reduction in the availability of specific nutrients. Coincidentally, our experiments detected a significant decrease in cell size before the onset of the stationary phase (Fig. 1).

If the hypothesis stated above could be extrapolated to all situations of unfavorable conditions, plasmid transfer between allochthonous bacteria in aquatic systems would acquire particular relevance. However, the results obtained for plasmid transfer during survival experiments in water from the River Butrón do not confirm the proposed hypothesis. As the time donor cells remain in water increases, the number of transconjugants decreases, and none are detected after 12 h under illuminated conditions or after 24 h under nonilluminated conditions. Given that both the coincubation conditions and physiological state of recipient cells remain constant, the variations in the numbers of transconjugants formed are attributable only to the physiological variations undergone by donor cells during their survival in river water.

Donor cells, however, lose their ability for plasmid transfer, although the parameters analyzed to detect variations in physiological state remain constant. Only in illuminated systems did we detect changes in the culturability and glucose uptake of strain EC_{15} after 48 and 24 h, respectively; before these changes took place, no transconjugants were detected after 8 h. According to these results, the parameters studied do not appear to have any relation to cell capacity for plasmid transfer during survival in river water microcosms. Transfer ability is probably more sensitive to environmental stress than to the variables analyzed in this work.

Moreover, this failure in the transfer process cannot be attributed to a partial or total loss of plasmids, since throughout the survival period we verified the culturability of cells on media supplemented with marker antibiotics. Likewise, Byrd and Colwell (10), Byrd et al. (11), Caldwell et al. (12), and Flint (15) have shown that plasmids are stably maintained in various hosts during long-term starvation in both river water and seawater. These authors did not study whether DNA can be transferred from these cells into a new host. Our results seem to indicate that the maintenance of plasmids by donor cells during their survival in freshwater systems does not necessarily imply a transfer of those plasmids. In an early study, Curtiss et al. (13) reported that F^+ and Hfr strains of *E. coli* lose more than 90% of their capacity to donate when cells are submitted to 6 h of starvation in saline solution. They observed that a loss of F pili was linked to donor ability. Goodman et al. (20) reported that transconjugants were detected from cultures of an *E. coli* donor strain for up to 3 days of prestarvation in artificial seawater but not after 5 days.

On the other hand, our results apparently disagree with those obtained by other authors. Oliver (31) observed that F^+ cells of *E. coli* remained able to conjugate, resulting in antibiotic-resistant exconjugants, for up to 48 days when donor cells were below the normal limits of detection by routine plating techniques. Garcia-Lara et al. (17) reported that *E. coli* populations retained their ability for conjugative plasmid transfer during 3 months of survival, even though they were not viable. These differences between results can probably be attributed to the different plasmids and bacterial species involved in transfer. In our case, the three donor strains show the same plasmid bands and present the same behavior with respect to transfer. It would be interesting to study the transfer of different plasmids in one strain throughout its survival.

Unlike the results obtained with donor cells, the capacity of recipient cells to receive and express plasmids did not vary during the time they remained in river water. In a similar way, Goodman et al. (20), working with *Vibrio* strains, obtained transconjugants when recipient cells were starved in artificial seawater for up to 100 days. Therefore, we must not forget when evaluating the risks involved with the transfer of plasmids between enterobacteria that although the capacity to donate decreases as time passes, it does remain constant for a reasonably long period, during which recipient cells retain their capacity to receive and express plasmids.

In any case, a global analysis of the results obtained allows us to deduce that during growth in culture media, the physiological variations undergone by donor cells have a significant influence on plasmid transfer frequency, while the same cannot be said for recipient cells under the same conditions. Likewise, environmental stress during the survival of cells in river water has a negative effect on the transfer capacity of donor cells but not on the capacity of recipient cells to receive and express plasmids.

ACKNOWLEDGMENTS

This study was supported by a predoctoral grant to M.P. and by research project PGV 9103, both from the Basque government, and by a predoctoral grant to J.I.J. from the Spanish government.

We thank two anonymous reviewers for their helpful suggestions, which have contributed to improving the quality of this work.

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