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# Camphor Plasmid-Mediated Chromosomal Transfer in *Pseudomonas putida*

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Camphor-utilizing strains of *Pseudomonas putida* have been shown to carry the genetic information required for camphor degradation on a plasmid. The plasmid-carrying strains can serve as donors of both plasmid-borne and chromosomal genes. As recipients, plasmid-deleted strains are much superior to those carrying the camphor pathway genes. The transfer frequency of chromosomal, but not plasmid-borne, genes is markedly enhanced if the donor cells are irradiated with ultraviolet light followed by 3-h of growth on a rich medium in the dark. Recombinants selected for prototrophy are stable and most acquire the camphor (CAM) plasmid concomitantly; only a few of the $Cam^+$ recombinants inherit the donor's ability to transfer chromosomal genes at a high frequency. Transfer-defective mutations occur on the CAM plasmid, affecting both CAM and chromosomal gene transfer.

Sexual conjugation is one of the well-known means of gene transfer among microorganisms (8). In all cases so far studied, mobilization of chromosomal genes occurs only from cells harboring some extrachromosomal element whose presence is essential for the donor action (8, 19). Among the plasmids known to initiate chromosome mobilization in *Escherichia coli* are the sex factor F, the colicin factors Col I and Col V, and the drug resistance factor R. In *Vibrio cholerae* (10), the fertility factor P is a plasmid associated with donor ability, as are in *Pseudomonas aeruginosa* (14, 23), the sex-factor FP and the drug-resistance factor R. Several other plasmids inducing chromosome mobilization have been described in other bacterial species (8).

Recently, we have demonstrated that the genes specifying enzymes responsible for the degradation of a number of organic compounds in *P. putida* are clustered together and are borne on transmissible plasmids (6, 7, 22). With positive evidence (5), but in the absence of a well-documented system for conjugational transfer of chromosomal genes in *P. putida*, we were interested in determining whether the degradative dissimilation plasmids would initiate an effective chromosomal gene transfer.

In this report, we present evidence that chromosomal genes are mobilized from *P. putida* cells harboring the gene cluster specifying camphor dissimilatory enzymes (CAM plasmid). We also were able to prepare mutants that are defective for the transfer of both plasmid and chromosomal genes. The responsible loci are on the plasmid. The conditions favoring the transfer of chromosomal markers at enhanced frequency have been explored and optimized in a preliminary way.

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## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The properties of donor cells harboring the CAM plasmid and the growth conditions for both donor and recipient to achieve plasmid transfer by conjugation have been described (7, 22). The strains used for chromosomal gene transfer and their derivation and properties are given in Table 1. Isolates used infrequently in these experiments have not received strain numbers in the Gunsalus collection; they lack PpG numbers.

**UV irradiation of the donor.** The cells of donor strains were grown overnight in L-broth on a shaker at 30 C. They were collected by centrifugation and suspended in 0.85% saline to the original culture volume. For irradiation, 5 ml (about $10^{10}$ cells/ml) was placed in a 5-cm petri dish and treated with a General Electric 15-W germicidal ultraviolet (UV) lamp from a distance of 35 cm for 0 to 60 s. Samples of the irradiated cells were inoculated into L-broth, and incubation was continued at 30 C for 3 h in the dark with shaking. The donor culture was then used for mating with a number of auxotrophic recipient strains.

TABLE 1. *Pseudomonas putida strains used*[a]

| PpG no. | Isolation no.[b] | Genotype | Phenotype | Parent | Treated | Reference |
|---|---|---|---|---|---|---|
| 1 | | wt/CAM | Cam$^+$ | | Camphor Enrichment | 22 |
| 273 | | *trpB615*/CAM | Trp$^-$Cam$^+$ | 1 | PC | 22 |
| 277 | | *trpB615*/CAM$^d$[c] | Trp$^-$Cam$^-$ | 273 | MC | 22 |
| 572 | | wt/CAM$^d$ | Cam$^-$ | 1 | PC | |
| 553 | | wt/Cam-121 | Cam$^-$ | | | |
| 566 | | wt/Cam-206 | Cam$^-$ | | | |
| 348 | | *met-601*/CAM$^d$ | Met$^-$Cam$^-$ | 1 | NG | |
| | 348 ArgB$^-$ | *met-601,argB000*/CAM$^d$ | Met$^-$Arg$^-$Cam$^-$ | 348 | NG | |
| | 572 Met$^-$ 1 | *Met-609*/CAM$^d$ | Met$^-$Cam$^-$ | 572 | NG | |
| | 572 Ilv$^-$ 1 | *Ilv-* /CAM$^d$ | Ilv$^-$Cam$^-$ | 572 | NG | |
| | 572 Ilv$^-$Trp$^-$ | *ilv-,trp-000*/CAM$^d$ | Ilv$^-$Trp$^-$Cam$^-$ | 572 | NG | |
| | 572 His$^-$ 1 | his-/CAM$^d$ | His$^-$Cam$^-$ | 572 | NG | |
| | 572 Arg$^-$ 1 | *arg-*/CAM$^d$ | Arg$^-$Cam$^-$ | 572 | NG | |
| 378 | 348 Cam$^+$ | *met-601*/CAM | Met$^-$Cam$^+$ | 273 × 348 | C | |
| | 348 ArgB$^+$Cam$^+$ | *met-601,argB000*/CAM$^d$ | Met$^-$Arg$^-$Cam$^-$ | 348 | NG | |
| | 572 Met$^-$Cam$^+$ | *met-609*/CAM | Met$^-$Cam$^-$ | 273 × 572 Met$^-$ | C | |
| | 572 Ilv$^-$Cam$^+$ | *ilv-* /CAM | Ilv$^-$Cam$^+$ | 273 × 572 Ilv$^-$ | C | |
| | 572 Ilv$^-$Trp$^+$Cam$^+$ | *ilv-* /CAM | Ilv$^-$Cam$^+$ | 273 × 572 Ilv$^-$Trp$^-$ | C | |
| | 572 Arg Cam$^+$ | *arg-* /CAM | Arg$^-$Cam$^+$ | 273 × 572 Arg$^-$ | C | |
| | H330 | *trpB615*/CAM$^d$ | Trp$^-$Cam$^+$ | 273 | NG | |
| | H331 | *trpB615*/CAM$^d$ | Trp$^-$Cam$^+$ | 273 | NG | |
| | H330 Tra$^+$ | *trpB615*/CAM | Trp$^-$Cam$^+$ | 273 × H330 CAM$^d$ | C | |

[a] Abbreviations: wt, prototroph; PC, penicillin-cycloserine; MC, mitomycin C; C, conjugation; NG, nitrosoguanidine.
[b] Isolates used infrequently were not assigned strain numbers.
[c] CAM$^d$—cured cells.
[d] Transfer defective mutants.

**Mating procedure.** The donor, with or without UV-irradiation, was grown for 3 h in the dark with shaking. The cell concentrations and transfer frequency varied according to treatment as outlined in the tables. The recipients were grown overnight in L-broth with shaking at 30 C to a cell density of $10^{10}$ to $2 \times 10^{10}$/ml. Equal volumes of donor and recipient cultures were mixed and allowed to stand at 30 C for 30 min without shaking. Samples were then plated on minimal or supplemented minimal plates to score for recombinants.

**Mitomycin C curing.** The procedure for mitomycin C curing was as described by Rheinwald et al. (22).

## RESULTS

The wide disparity in transfer frequency of plasmid- versus chromosome-borne markers in *P. putida* and related fluorescent pseudomonads, about $10^{-3}$ versus $10^{-8}$ per donor in plasmid-mediated conjugation, led us to seek methods of enhancing the frequency for chomosomal markers. In *E. coli*, a range of donor effectiveness in conjugation is known, as are some procedures for improving the capacity in sluggish donors (13, 21). Our experiments, successful in enhancing chromosome transfer, are recorded here. The relevance of the octane (7), naphthalene (12), and salicylate (6) plasmids were the subject of additional studies to be recorded elsewhere.

**Conditions for transfer of chromosomal genes.** In *P. putida*, UV irradiation followed by an outgrowth period enhances the transfer of chromosomal, but not plasmid-borne, markers (Fig. 1 and 2; Table 2). The conjugation is associated with the presence of extrachromosomal elements, bearing genes for peripheral metabolic enzymes, and with plasmid loci affecting the transfer of chromosomal genes. The transfer frequency is variable among strains for both plasmid and chromosomal genes. Thus, the behavior resembles that in *E. coli* where the sex factor F produces chromosomal recombinants at a frequency of $10^{-4}$ to $10^{-5}$ per donor, whereas the colicin factors Col I and V and the drug resistance factor Rl produce recombinants at only $10^{-7}$ to $10^{-8}$ per donor. The low recombination frequency found for the colicins and for R factor have usually been ascribed to a defective rate of plasmid integration with the chromosome or to repression of the conjugation process itself (15, 17). With *P. putida* carrying the CAM plasmid, the number of recombinants for several chromosomal markers is around one per $10^8$ to $10^9$ donors (Table 2). The results in Table 2 and Fig. 1 show that UV irradiation increases the recombination frequency for most chromosomal markers. In view of the well-known ability of UV irradiation to enhance genetic integration (2, 3), it seems possible that this increase

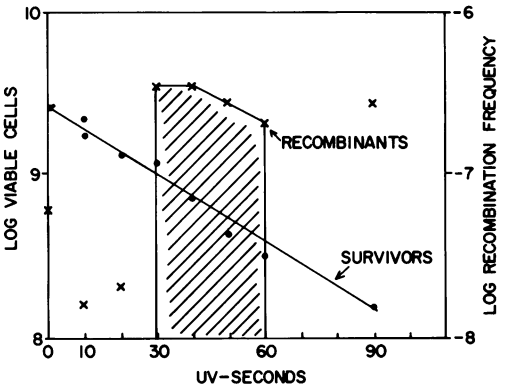


FIG. 1. *Influence of UV irradiation on recombination frequency. Late log-phase cultures of PPG273 were UV irradiated (see Materials and Methods) and grown in the dark for 3 h. Samples irradiated at intervals (×) from 0 to 90 s were titered for survivors and mated to an auxotrophic recipient, 572 Met⁻.*

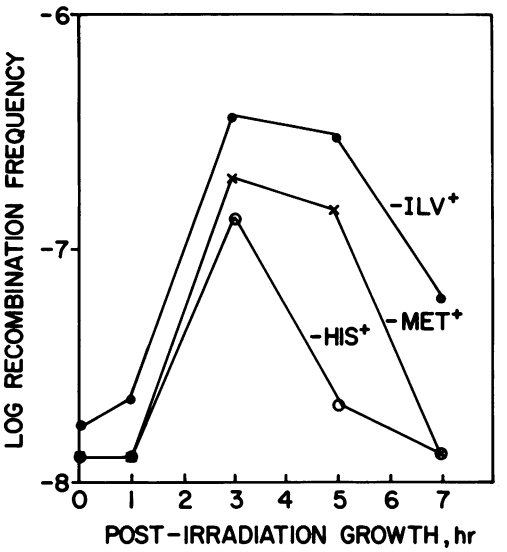


FIG. 2. *Chromosomal marker transfer enhanced by postirradiation growth. PPG273, UV irradiated for 30 s, was grown in the dark. Matings with indicated auxotrophs, as shown in Materials and Methods.*

may result from a more frequent recombination of part, or all, of the CAM plasmid at different chromosomal sites in *P. putida* strain PpG1.

With the enhanced formation of recombinants for chromosomal markers, we inquired whether irradiation leads to an enhanced frequency of CAM plasmid transfer. The data in Table 2 show clearly that UV irradiation does not stimulate CAM plasmid infection in the recipients. The frequency of CAM transfer, which usually remains unaltered at low UV dosage, may even decrease at higher doses. Since the rate of elimination of the CAM plasmid is not increased by UV treatment (22), this decrease is presumably not due to curing the donor cells of this plasmid.

The increased frequency of gene transfer induced by UV has been well documented in the case of the *E. coli* sex factor F, where the maximal effect is observed only after the cells are allowed to grow for 45 to 90 min after irradiation (13, 21). In *P. putida*, the frequency of genetic recombination for a number of chromosomal genes reaches a peak only when the irradiated donor cells are grown for 3 h in L-broth in the dark (Fig. 2). Growth for shorter or longer periods was less effective in increasing recombinant yield; this suggests that the maximal potential for gene transfer is dependent on a particular stage of growth of the irradiated donor cells.

**Recombinant properties.** All the recombinants selected on minimal plates appear to be stable prototrophs, and 90 to 95% are also Cam⁺. To determine whether the Cam⁺ character is accompanied by integration of the plasmid into the recipient genome, several Cam⁺ recombinants were purified by single-colony isolation and treated with mitomycin C to test for curing of the CAM plasmid (22). The treated cells were then plated on L-agar, and single colonies were replica-plated to camphor and glucose minimal plates to determine whether elimination of the CAM plasmid might be

TABLE 2. *Irradiation and chromosomal versus plasmid transfer*

| Recipient[a] | Selected | Recombinants/$10^8$ donors | | | Recombinants/$10^4$ donors | | |
|---|---|---|---|---|---|---|---|
| | | 0 s[b] | 30 s | 60 s | 0 s | 30 s | 60 s |
| Chromosomal | | | | | | | |
| 572 Ilv⁻ | Ilv⁺ | 4 | 36 | 80 | | | |
| 572 His⁻ | His⁺ | 9 | 40 | 88 | | | |
| 572 Met⁻ | Met⁺ | 8 | 60 | 40 | | | |
| 572 Arg⁻ | Arg⁺ | 0.1[c] | 30 | 40 | | | |
| 348 Met⁻Arg⁻ | Met⁺ | 2 | 20 | 40 | | | |
| 348 Met⁻Arg⁻ | Arg⁺ | 2 | 8 | 26 | | | |
| Plasmid | | | | | | | |
| 572 Cam[d] | Cam⁺ | | | | 30 | 20 | 10 |
| 553 Cam-121 | Cam⁺ | | | | 3 | 2 | 2 |
| 566 Cam-206 | Cam⁺ | | | | 16 | 4 | 2 |

[a] Donor was PPG273 = *trpB615*/CAM.

[b] Time of UV irradiation. Revision influences were about $10^{-9}$.

[c] *N* spontaneous rate.

accompanied by the loss of the chromosomal gene fragment. In all cases, loss of the Cam$^+$ character occurred without concomitant loss of the chromosomal markers (Table 3). Thus, the recombinants seem to have acquired the Cam$^+$ character by secondary infection. We have been unable to detect loss of chromosomal markers under conditions that wholly eliminate CAM, suggesting that the recombinants are haploid cells with donor chromosomal fragments integrated into the chromosome.

**Plasmid (CAM) effect on chromosome transfer.** The presence of the CAM plasmid inside the donor is essential for effective chromosome mobilization. In recipients, the presence of the plasmid reduces or eliminates expression of chromosome transfer. This can be seen more clearly from the data in Table 4, where the results of several crosses involving Cam$^+$ and Cam$^-$ donors and recipients are presented. In these experiments, elimination of the CAM plasmid from the donor eliminated transfer of chromosomal genes, i.e., to $<10^{-8}$/donor. The recipients used in such crosses are all Cam$^-$. If Cam$^+$ recipients are used, the transfer frequency for chromosomal genes is eliminated or sharply reduced, irrespective of the Cam$^+$ nature of the donor. It is thus clear that not only the presence of the CAM plasmid is essential in the donor for detectable transfer of chromosomal markers, but its presence in a recipient prevents chromosomal transfer. The latter may arise either from exclusion of entry or from loss of expression of the chromosomal fragment. The transfer of CAM plasmid genes is affected by the presence of the plasmid in recipients (Table 2, see also reference 22) but to a far lesser degree than the transfer of chromosomal markers.

**Chromosome mobilization by recombinants.** If chromosome mobilization induced by the CAM plasmid is due to integration of part of the CAM plasmid with the donor chromosome, one might expect some recombinants to acquire integrated plasmid genes along with the chromosomal fragment and become high-frequency donors. A number of recombinants from each of several crosses were therefore tested for their ability to transfer chromosomal markers to other recipients. It can be seen from the results in Table 5 that these recombinants can, in fact, transfer chromosomal markers. The frequency appears to be a function of the donor and the auxotrophic marker tested in the recipient. The latter was also found with the parent donor, 273, as shown in Table 2. The ability of Cam$^+$ exconjugants constructed from Cam$^-$ plasmid cured strains to act as genetic donors again indicates that the donor ability is associated with the presence of the CAM plasmid. Some of the recombinants transfer chromosomal markers at the higher frequency of the parent Cam$^+$ donors but only after UV irradiation and outgrowth. Thus, in the respect to the UV requirement they behave as the parent donors.

**Transfer defective loci on CAM plasmid.** The essentiality of the CAM plasmid for chromosome mobilization, and the enhanced trans-

TABLE 3. *Mitomycin C curing of CAM from recombinants*

| Cross | Recombinant phenotype | Exconjugant ratio (Cam$^-$/total) | | |
|---|---|---|---|---|
| | | Untreated | MC[a], (10 μg/ml) | Phenotype |
| 273 × 572 Met$^-$ | Met$^+$Cam$^+$ | 0/108 | 96/96 | Met$^+$Cam$^-$ |
| 273 × 572 Met$^-$ | Met$^+$Cam$^+$ | 2/66 | 123/123 | Met$^+$Cam$^-$ |
| 273 × 572 His$^-$ | His$^+$Cam$^+$ | 2/70 | 93/93 | His$^+$Cam$^-$ |

[a] MC, Mitomycin C.

TABLE 4. *CAM plasmid effect in donor versus recipient*[a]

| Donor No. | Donor Phenotype | Recipient Cam genotype No. Phenotype | CAM[a] 348 Met$^-$ | CAM[a] 572 Met$^-$ Met$^-$ | CAM[a] 572 His$^-$ His$^-$ | CAM 348 Met$^-$ | CAM 572 Met$^-$ Met$^-$ | CAM 572 Arg$^-$ Arg$^-$ |
|---|---|---|---|---|---|---|---|---|
| 273 | Trp$^-$Cam$^+$ | | 100[b] | 50 | 38 | <2 | <2 | <2 |
| 277 | Trp$^-$Cam$^-$ | | <2 | <2 | <2 | | | |
| 572 Ilv$^-$Cam$^+$ | Ilv$^-$Cam$^+$ | | 500 | | | | | |
| 572 Ilv$^-$ | Ilv$^-$Cam$^-$ | | <2 | | | | | |

[a] Recombinants selected for prototrophy. Mating as described in Materials and Methods with 30 s of UV and followed by 3 h of growth at 30 C in L-broth in the dark.

[b] Number of recombinants per $10^8$ donors.

fer frequency for chromosomal markers after UV irradiation signify some interaction, stable or unstable, between the CAM plasmid and the donor chromosome. It is not clear if the genes responsible for the CAM plasmid transfer are the same ones that participate in mobilization of the chromosomal markers. To determine whether common genetic determinants affect both functions, transfer-defective mutants were isolated from cells harboring the CAM plasmid. Such mutants transfer the CAM plasmid at a reduced frequency (Table 6), e.g., about $10^{-6}$ compared to $10^{-3}$ for the parent (Table 2). Interestingly, the transfer frequency for chromosomal genes is also severely depressed in these mutants. When the CAM plasmid is removed from such transfer-defective mutants by treatment with mitomycin C and a wild-type CAM plasmid is introduced, e.g., H330 Tra$^+$ (Table 6), the usual transfer frequency is restored for both the plasmid and the chromosomal markers, suggesting that the transfer-defective mutation was on the plasmid. Further study of these transfer-defective mutations and, as well, attempts to map their loci are presently in progress in our laboratory.

## DISCUSSION

The low frequency of chromosomal gene mobilization in *P. putida* strain PpG1 carrying the CAM plasmid resembles the rate in *E. coli* carrying the Col and R factors rather than F. That is, F mobilizes chromosomal markers at a frequency at $10^{-4}$ to $10^{-5}$ per donor, whereas Col I and most R factors give frequencies varying from $10^{-7}$ to $10^{-9}$ (8, 20). These differences are due to formation of repressor elements that prevent pilus synthesis and thus decrease conjugation frequency (17). The drug resistance factor R1 has been shown to have a specific affinity for integration with the chromosomal *trp* genes, forming an F-like derivative of the plasmid (20). In CAM the low-expression frequency of chromosomal genes might result from a low integration rate of the plasmid with the host chromosome. It is usually assumed that the transfer of chromosomal markers by an F$^+$ population is due to the presence of Hfr derivatives formed by the integration of F with the host chromosome (4, 8). It is possible, however, that integration may not be a prerequisite for chromosome transfer by the fertility factors, and there might exist other mechanisms whereby transfer of chromosomal genes can take place (9).

In the case of CAM plasmid, the low frequency of transfer of chromosomal markers may, therefore, simply be a reflection of low rate of integration of the plasmid genes with the host chromosome. Since UV irradiation is known to enhance genetic recombination, the increased frequency of chromosomal gene transfer after UV irradiation of the donor could result from more frequent integration of the plasmid genes with the chromosome (2, 3). The low-frequency donor ability of the recombinants would be similar to the F′ nature of recombinants formed in Hfr × F′ crosses. On the other hand, it is equally possible that CAM and chromosome transfer are independent events and that no portion of the CAM plasmid need be integrated with the host chromosome to effect chromosome transfer. Thus, the increased chromosomal marker transfer frequency after UV irradiation would be due to formation of unstable recombinants, as postulated by Evenchik et al. (13), where one single strand of the CAM plasmid would be joined with one strand of the chromosome to give a temporary inheritable donor state capable of transferring the chromosome to the recipients. Present data do not distinguish be-

TABLE 5. *Chromosome mobilization by recombinants*

| Donor | Recipient | Sel. | Recombinants/ $10^8$ donors | | |
|---|---|---|---|---|---|
| | | | 0 s[a] | 30 s | 60 s |
| 572 Ilv$^-$Trp$^+$Cam$^+$ | 572 Met$^-$ | Met$^+$ | 48 | 160 | 320 |
| 572 Ilv$^-$Trp$^+$Cam$^+$ | 572 Arg$^-$ | Arg$^+$ | 3 | 20 | 44 |
| 348 Met$^-$Arg$^+$Cam$^+$ | 572 Met$^-$ | Met$^+$ | 2 | 9 | 16 |
| 348 Met$^-$Arg$^+$Cam$^+$ | 572 Arg$^-$ | Arg$^+$ | 4 | 5 | 20 |

[a] Time of UV irradiation.

TABLE 6. *Chromosomal and plasmid (/CAM) transfer defective mutants*

| No. | Donor[a] Phenotype | Recipient No. Phenotype | 572 wt Cam$^-$ | 572 Met$^-$ Met$^-$Cam$^-$ | 572 Ilv$^-$ Ilv$^-$Cam$^-$ | 572 Arg$^-$ Arg$^-$Cam$^-$ |
|---|---|---|---|---|---|---|
| H330[b] Tra$^+$ | Tra$^+$Trp$^-$Cam$^+$ | | 10,000[c] | 20 | 6 | 4 |
| H330 | Tra$^-$Trp$^-$Cam$^+$ | | 100 | <2 | <2 | <2 |
| H331 | Tra$^-$Trp$^-$Cam$^+$ | | 100 | <2 | <2 | <2 |

[a] Irradiated for 30 s, followed by 3 h of growth at 30 C in L broth in dark before mating.
[b] Wild-type CAM was introduced into a Cam$^-$ derivative of H330.
[c] Number of recombinants per $10^8$ donors.

tween these two mechanisms of chromosome mobilization from Cam$^+$ donor cells.

The presence of a CAM plasmid in the recipient drastically reduces the frequency of gene transfer. A negative interaction among plasmids is not uncommon and has been compared to superinfection immunity between closely related phages (18, 24). This plasmid immunity might be due either to the exclusion in entry of the donor plasmid to plasmid bearing recipients, which is quite independent of sex piliation and plasmid incompatibility (1, 25), or it might also be due to lack of maintenance and establishment of the incoming plasmid even when the entry barrier has been removed (11, 19). We have not at this time determined at which level the interference in gene expression occurs with Cam$^+$ recipients. Interestingly, prototrophic Cam$^+$ recombinants are recoverable from crosses of Trp$^-$Cam$^+$ donors and prototrophic Cam$^-$ point mutants (A. M. Chakrabarty and I. C. Gunsalus, Bacteriol. Proc. 1971, p. 46). This most probably results from the entry, synapsis, and recombinational events between the resident and the donor plasmid. This would signify that an inherent incompatibility, rather than an entry exclusion, is involved in the phenomenon of CAM-induced decrease in gene acceptance and expression among recipients. Since the frequency of CAM transfer is much higher than the transfer of chromosomal genes, almost all the recombinants acquire CAM by secondary infection.

The inability of the transfer-defective Cam$^+$ mutant cells to transfer either chromosomal or CAM plasmid markers indicates some correlation between the two processes. The transfer-defective mutants of both F$^-$ and Col I plasmids have been extensively studied in *E. coli* by complementation analysis, and at least 12 cistrons have been found to effect F transfer (1, 25). Since the frequency of transfer-defective mutations in the CAM plasmid is rather high, it is conceivable that several genes are involved in the transfer. Attempts to identify CAM-specific surface antigens or pilus-like structures have so far been negative. It would be interesting to map transfer-defective mutations on the CAM plasmid and determine the number of cistrons involved specifically in CAM transfer and in chromosome mobilization.

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