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Utilization of a Thermosensitive Episome Bearing Transposon Tn*10* to Isolate Hfr Donor Strains of *Erwinia carotovora* subsp. *chrysanthemi*

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A thermosensitive episome bearing the transposon Tn*10*, F(Ts)::Tn*10* Lac$^+$, has been successfully transferred from *Escherichia coli* to several wild strains of the enterobacteria *Erwinia carotovora* subsp. *chrysanthemi*, which are pathogenic on *Saintpaulia ionantha*. In one of these strains, all of the characters controlled by this episome (Lac$^+$, Tet^r, Tra$^+$) were expressed, and its replication was stopped at 40°C and above. At 30°C, the episome was easily transferred between strains derived from *E. carotovora* subsp. *chrysanthemi* 3937j and to *E coli*. Hfr donor strains were obtained from a F′ strain of 3937j by selecting clones which grew at 40°C on plates containing tetracycline. One of these strains, Hfrq, was examined in more detail: the characters Lac$^+$ and Tet^r were stabilized and did not segregate in Hfrq, and this donor was able to transfer Leu$^+$ at a frequency 1,000 times higher than its parental F′ strain. The mating was most efficient at 37°C on a membrane. Hfrq transferred its chromosome to recipient strains at high frequency and in a polarized fashion, as evidenced by the gradient of transfer frequencies, the kinetics of marker entry (in interrupted mating experiments), and the analysis of linkage between different markers. The chromosome of Hfrq was most probably transferred in the following sequence: origin. . .*met*. . .*xyl*. . .*arg*. . . *ile*. . .*leu*. . .*thr*. . .*cys*. . .*pan*. . .*ura*. . .*gal*. . .*trp*. . .*his*. . .*pur*. . . Moreover, this genetic transfer system proved to be efficient in strain construction.

Erwinia carotovora subsp. *chrysanthemi* (12) is an enterobacterium pathogenic for several monocotyledonous and dicotyledonous plants (14, 19, 20, 25). In most cases (but not all) the infection is characterized by a soft-rot symptom for which secretion by the bacteria of an endo-polygalacturonate-*trans*-eliminase is necessary (4, 8, 13). In addition, *E. carotovora* subsp. *chrysanthemi* phytopathogenic strains present a host specificity (19, 20).

To study the genetic determinants of the pathogenicity, we have attempted to obtain a chromosomal transfer system in a strain of *E. carotovora* subsp. *chrysanthemi* which is pathogenic for *Saintpaulia ionantha*. On this plant, soft-rot symptoms elicited by the bacteria appear 2 days after the onset of infection. Moreover, at 30°C and under strong hygrometry, the infected plants died in less than 10 days (20). The test for the pathogenic power gives a quick and unambiguous answer. Furthermore, the taxonomic proximity of *Escherichia coli* and *E. carotovora* subsp. *chrysanthemi* has already allowed transfer of an F′Lac$^+$ episome to this bacterium (7) and derivation of an Hfr donor strain from it (8).

In *Salmonella typhimurium* a thermosensitive episome bearing the transposon Tn*10*, which determines an inducible resistance to tetracycline (17) [F′(Ts)::Tn*10* Lac$^+$], has been used to construct Hfr strains with known origins and directions of transfer (10, 18). This was done by taking advantage of (i) the thermosensitive replication of the episome, and (ii) the integration of the episome by recombination between the copy of the Tn*10* on the F′ and another copy already present on the chromosome.

Here, we report a method for isolation of Hfr strains of *E. carotovora* subsp. *chrysanthemi* which can transfer their chromosomal genes at frequencies similar to those observed in *E. coli*. The properties of the transfer system were studied in one of these Hfr strains, and a sequence of chromosomal markers was determined.

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MATERIALS AND METHODS

Bacterial strains and bacteriophages. Strains of *E. carotovora* subsp. *chrysanthemi* and *E. coli* used are listed in Table 1. Bacteriophages R17 and Qβ were from our collection.

Culture media. The complex medium usually used was LB medium (22). The indicator medium for lactose fermentation (TTC3 Lac) was LB medium solidified by 15 g of agar per liter; 50 mg of 2,3,5,-triphenyl tetrazolium chloride per liter was added before autoclaving, and 50 ml of a sterile solution containing 200 mg of lactose per ml was added after autoclaving. The minimal medium was the A medium of Miller (22). The carbon source was added after autoclaving at a final concentration of 2 g/liter. The amino acids were added in their L form at a concentration of 30 μg/ml.

Antibiotics. For *E. carotovora* subsp. *chrysanthemi*, streptomycin was added at the final concentration of 500 μg/ml, tetracycline was added at 15 μg/ml, rifampicin was added at 100 μg/ml, and nalidixic acid was added at 20 μg/ml.

Culture temperature. *E. carotovora* subsp. *chrysanthemi* and *E. coli* (bearing the thermosensitive episome) were grown at 30°C.

Mutagenesis of *E. carotovora* subsp. *chrysanthemi*. The chemical mutagenesis method with ethyl methane sulfonate (22) was modified as follows: bacteria were grown overnight in 10 ml of A medium supplemented with glucose in a reciprocal shaker at 30°C. They were then diluted 10-fold in the same medium and grown for 3 or 4 h until the concentration reached 5×10^8 bacteria per ml. The cells were then centrifuged, washed, and suspended in half the initial volume of A medium without glucose. Ethyl methane sulfonate was added at 15 μl/ml and incubated for 2 h at 30°C. Then the mixture was diluted 100-fold (0.1 ml into 10 ml of LB medium) and grown overnight to allow the phenotypic expression of mutations.

Ampicillin treatment. The ampicillin treatment was adapted from Miller (22) as follows. After the phenotypic expression of mutations, the culture was centrifuged, washed two times in buffer, and diluted 1,000-fold (10 μl into 10 ml) in A medium plus glucose. After 3 to 4 h of cultivation, ampicillin was added to a final concentration of 20 μg/ml. After overnight treatment in a shaking water bath, the contents of the flasks were centrifuged at 10^4 rpm for 20 min. The pellets were suspended in 10 ml of LB medium, and the cultures were grown with agitation at 30°C until they reached the stationary phase.

Isolation and characterization of auxotrophic mutants. We used Holliday's method (16) for isolation and characterization of auxotrophic mutants. The cultures were spread on complex medium; after incubation for 48 h, the plates were replicated on minimal medium plus glucose and on LB plates. Suspensions from colonies of auxotrophic mutants were prepared, and drops were put on plates containing the pools of amino acids, bases, and vitamins. After purification, the frequency of spontaneous reversion of mutants selected was measured.

Conjugation conditions. Crosses were done in liquid or solid medium at 30°C (except when the donor was an Hfr strain; see below). LB medium cultures in log phase were used for the conjugation mixture. The conjugation ratio (number of donor cells per recipient cell) was between 1/5 and 1/20. In liquid medium, conjugation occurred in a water bath with slow shaking. Filter mating was performed by filtering the conjugation mixture through a Millipore membrane (pore size, 0.22 μm); the membrane was then layered on a plate of rich medium containing 2 g of glucose per liter prewarmed at the conjugation temperature. Conjugation took place for 2 to 6 h. Controls were treated in the same way. In the case of crosses with an Hfr donor strain of *E. carotovora* subsp. *chrysanthemi*, the conjugation was for 2 h at 37°C on membranes. At the end of the mating, the membrane was introduced into a tube containing 2 ml of buffer, and the cells were resuspended by strong agitation for 1 min on a tube shaker.

Interrupted mating. Interrupted mating was done at 30°C, on membranes by the technique of Chatterjee and Starr (8); a filter mating was simultaneously performed on several membranes and was interrupted by shaking each membrane in buffer at a different time after the beginning of the mating.

RESULTS

Transfer of the episome into *E. carotovora* subsp. *chrysanthemi*. Seven wild-type strains of *E. carotovora* subsp. *chrysanthemi* isolated from *Saintpaulia* were used as recipients in crosses with strain 6895 of *E. coli*, which harbors the episome F′(Ts)::Tn*10* Lac^+. The matings were performed on membranes, and transconjugants were scored on minimal medium containing tetracycline (Table 2). In the rest of the study, strain 3937j was chosen because it expressed a stronger virulence on *Saintpaulia* than did the strain 3937b (data not shown). The transconjugants of *E. carotovora* subsp. *chrysanthemi* strain 3937j expressed a Lac^+ phenotype (i.e., the colonies appeared after 2 to 3 days on minimum medium with lactose as the carbon source, and they were white on indicator medium TTC3Lac at 30°C), whereas the parental F^- strain was phenotypically Lac^- (i.e., the colonies took 10 days to grow on minimal medium with lactose, and they were red on TTC3Lac medium). At 30°C, the transconjugants were resistant to tetracycline up to at least 25 μg/ml, and this resistance was inducible like that mediated by the transposon Tn*10* (17). The sensitivity of the transconjugants to the bacteriophages R17 and Qβ was checked by cross-streaking; the sensitivity was stable at 34°C, but disappeared at 40°C. A total of 100 colonies of strain 3937jN(F′), which were grown at 40°C on rich and nonselective medium (LB medium without tetracycline), were picked up and tested for the following characters: resistance to tetracycline (Tet^r), sensitivity to phage R17 (Tra^+) at 34°C, and lactose fermentation (Lac^+); all of them were Tet^s, Tra^-, and Lac^- and therefore probably F^-. Thus, the thermosensitivity of the episome

TABLE 1. Bacterial strains

Strain	Genotype or phenotype[a]	Source
E. carotovora subsp. *chrysanthemi*		
3902	Wild-type, isolated from *Saintpaulia*	Our collection
3918	Wild-type, isolated from *Saintpaulia*	Our collection
3920	Wild-type, isolated from *Saintpaulia*	Our collection
3929	Wild-type, isolated from *Saintpaulia*	Our collection
3937j	Wild-type, isolated from *Saintpaulia*	Our collection
3937b	Wild-type, isolated from *Saintpaulia*	Our collection
3942	Wild-type, isolated from *Saintpaulia*	Our collection
3920S	Str^r, prototrophic	Spontaneous mutant of 3920
3920R	Rif^r, prototrophic	Spontaneous mutant of 3920
3937jN	Nal^r, prototrophic	Spontaneous mutant of 3937j
3902(F′)	Lac^+ Tet^r Tra^+	3902 carrying the episome F′(Ts114)::Tn*10* Lac^+
3918(F′)	Lac^+ Tet^r Tra^+	3918 carrying the episome F′(Ts114)::Tn*10* Lac^+
3929(F′)	Lac^+ Tet^r Tra^+	3929 carrying the episome F′(Ts114)::Tn*10* Lac^+
3937b(F′)	Lac^+ Tet^r Tra^+	3937b carrying the episome F′(Ts114)::Tn*10* Lac^+
3937jN(F′)	Lac^+ Tet^r Tra^+ Nal^r	3937jN carrying the episome F′(Ts114)::Tn*10* Lac^+
3920R(F′)	Lac^+ Tet^r Tra^- Rif^r	3920R carrying the episome F′(Ts114)::Tn*10* Lac^+
AK370	Str^r, prototrophic	Spontaneous mutant of 3937j
AK371	*arg-1* Str^r	EMS[b] mutagenesis of AK370
AK372	*his-1* Str^r	EMS mutagenesis of AK370
AK373	*leu-1* Str^r	EMS mutagenesis of AK370
AK381	*met-1* Str^r	EMS mutagenesis of AK370
AK384	*xyl-1* Str^r	EMS mutagenesis of AK370
AK3710	*arg-1 ile-2* Str^r	EMS mutagenesis of AK371
AK3711	*arg-1 met-3* Str^r	EMS mutagenesis of AK371
AK3712	*arg-1 thr-2* Str^r	EMS mutagenesis of AK371
AK3716	*arg-1 leu-1* Str^r	This paper, recombinant of H331 × AK3712
AK3720	*his-1 gal-1* Str^r	EMS mutagenesis of AK372
AK3727	*his-1 trp-1* Str^r	EMS mutagenesis of AK372
AK3731	*leu-1 ile-4* Str^r	EMS mutagenesis of AK373
AK3733	*leu-1 his-2* Str^r	EMS mutagenesis of AK373
AK3734	*leu-1 thr-3* Str^r	EMS mutagenesis of AK373
AK3812	*met-1 leu-3* Str^r	EMS mutagenesis of AK381
AK3841	*xyl-1 met-7* Str^r	EMS mutagenesis of AK384
AK3842	*xyl-1 trp-3* Str^r	EMS mutagenesis of AK384
AK3845	*xyl-1 leu-1* Str^r	This paper, recombinant of H311 × AK3842
AK37163	*arg-1 leu-1 cys-4* Str^r	EMS mutagenesis of AK3716
AK37164	*arg-1 leu-1 met-8* Str^r	EMS mutagenesis of AK3716
AK37166	*arg-1 leu-1 ura-2* Str^r	EMS mutagenesis of AK3716
AK37345	*leu-1 thr-3 pur-3* Str^r	EMS mutagenesis of AK3734

TABLE 1—*Continued*

Strain	Genotype or phenotype[a]	Source
AK38452	*xyl-1 leu-1 ile-6* Str^r	EMS mutagenesis of AK3845
AK38454	*xyl-1 leu-1 pan-2* Str^r	EMS mutagenesis of AK3845
AK38455	*xyl-1 leu-1 met-10* Str^r	EMS mutagenesis of AK3845
Hfrq	Hfr Lac$^+$ Tet^r Tra$^+$ Nal^r, prototrophic	This paper, derived from 3937jN(F′)
H311	Hfr Lac$^+$ Tet^r Tra$^+$ Str^r, *leu-1 ile-4*	This paper, derived from AK3731
H331	Hfr Lac$^+$ Tet^r Tra$^+$ Str^r, *leu-1 his-2*	This paper, derived from AK3733
E. coli		
C600	rK$^-$ Thr$^-$ Leu$^-$ Str^r	Our collection
NK5222	His$^-$ Spc^{r}ΔXIII(*lac-pro*)	N. Kleckner
6895	*argE*(Am) *metB* Rif^r Nal^r Δ(*lac-pro-sul*)/F′(Ts114) Lac$^+$::Tn*10*	D. Botstein

[a] The abbreviations used to design the markers are according to Bachmann and Low (1). The Ile$^-$ strains were isolated as mutants unable to grow in the absence of isoleucine.
[b] EMS, Ethyl methane sulfonate.

TABLE 2. Transfer of the episome F(Ts)::Tn*10* Lac$^+$ from *E. coli* 6895 to several strains of *E. carotovora* subsp. *chrysanthemi* and *E. coli*[a]

Recipient strain	Transfer frequency of Tet^r [b]
E. carotovora subsp. *chrysanthemi*	
3902	5.9×10^{-6}
3918	1.2×10^{-6}
3920	1.5×10^{-5}
3929	6.5×10^{-5}
3937b	6.6×10^{-3}
3937j	3.3×10^{-3}
3942	$<10^{-7}$
E. coli	
NK5222	3.6×10^{-1}

[a] Matings were done on membranes at 30°C as described in the text. Controls treated in the same way were negative.
[b] Number of Tet^r recipient cells per input donor cell of *E. coli* 6895.

seems to be expressed at 40°C in *E. carotovora* subsp. *chrysanthemi* 3937jN(F′).

We studied the stability of the plasmid in strain 3937jN(F′) at 30°C by picking up three white colonies grown for 72 h on indicator medium TTC3Lac; after suspension, dilution, spreading, and incubation on indicator medium TTC3Lac, it appeared that, among the bacteria deriving from the white colonies, an average of 26% were Lac$^-$ (they gave red colonies on indicator medium).

Behavior of *E. carotovora* subsp. *chrysanthemi* as donor of the episome. The results of the transfer of the F′ factor from *E. carotovora* subsp. *chrysanthemi* to *E. coli* and to *E. carotovora* subsp. *chrysanthemi* are shown in Table 3. The F′ episome was transferred by conjugation from the strains 3902(F′), 3918(F′), 3920R(F′), 3929(F′), 3937b(F′), and 3937jN(F′) to *E. carotovora* subsp. *chrysanthemi* 3937j and 3920S and to a restriction-deficient derivative of *E. coli* C600. The frequencies of transfer observed are very similar to those obtained in the mating between *E. coli* 6895 and NK5222, except when *E. carotovora* subsp. *chrysanthemi* is the donor or the recipient (or both). The transfer from the donor strains 3937b(F′) and 3937j(F′) was as frequent to *E. coli* as to *E. carotovora* subsp. *chrysanthemi* 3937j.

Incidentally, strain 3920R(F′) was mated at 30°C with *E. coli* strains NK5222 and C600 in liquid medium; the transfer frequency of the F′ factor was 2.6×10^{-6} with NK5222 and 4.8×10^{-4} with C600.

Isolation of strains able to transfer chromosomal markers. A fresh culture of strain 3937jN(F′) was diluted, and several dilutions

TABLE 3. Donor ability of *E. carotovora* subsp. *chrysanthemi* F' strains[a]

Donor	Recipient	Frequency of Tet^r transfer (Tet^r recipients/ input donor cell)	Sensitivity of donor to phages[b]	
			Qβ	R17
E. carotovora subsp. *chrysanthemi*	*E. coli*			
3902(F')	C600	4.9×10^{-1}	+	+
3918(F')	C600	4.0×10^{-1}	+	+
3920R(F')	C600	8.1×10^{-6}	−	−
3929(F')	C600	4.1×10^{-1}	+	+
3937b(F')	C600	7.4×10^{-1}	+	+
3937jN(F')	C600	1.1×10^{-2}	+	NT
E. carotovora subsp. *chrysanthemi*	*E. carotovora* subsp. *chrysanthemi*			
3920R(F')	3920S	6.2×10^{-8}		
3937b(F')	3920S	8.0×10^{-4}		
3937b(F')	3937jN	1.4×10^{-1}		
3937jN(F')	3920S	3.0×10^{-5}		
3937jN(F')	AK370	9.1×10^{-3}		

[a] Crosses were performed on membranes as described in the text.
[b] Sensitivity to the male-specific bacteriophages was tested by cross-streaking at 34°C. NT, Not tested.

were spread on plates of indicator medium TTC3Lac containing 15 μg of tetracycline per ml. Plates were incubated at 40°C; after 4 days small colonies appeared on the plates. The number of these colonies represented about 10% of those growing at the same temperature on indicator medium without tetracycline. A total of 336 colonies which were Tet^r at 40°C were picked up, gridded, and tested for several characters by replica plating; they were all Lac$^+$ at 30 and 40°C (growth was observed on minimal medium with lactose as carbone source) and also Tet^r at 30 and 40°C. Furthermore, all colonies were prototrophic and resistant to nalidixic acid, as was the parental strain.

Later on, 20 clones stemming from the 336 colonies previously examined were purified three times at 40°C on the selection medium and tested by cross-streaking for sensitivity to the male-specific bacteriophage R17 at 34 and 40°C; 11 were sensitive to the phage at both temperatures, whereas the others were resistant at both temperatures. The 11 R17-sensitive clones were examined with a qualitative method for their ability to transfer chromosomal markers (drops of the mating mixtures were layered on nonselective rich medium and allowed to dry, and after overnight incubation at 30°C, recombinants were scored by replica plating on selective media). They all transferred Arg$^+$, His$^+$, Leu$^+$, Ade$^+$, Ura$^+$, and Lys$^+$ at 30°C (data not shown). One of these donor strains, Hfrq, was studied in more detail.

Properties of the donor strain Hfrq. Strain Hfrq was very stable; no segregation or simultaneous loss of Lac$^+$ and Tet^r characters was observed when it was grown in liquid or solid medium in absence of selective pressure (i.e., in the absence of tetracycline or lactose as the sole carbon source). When purified on indicator medium TTC3Lac, Hfrq gave only white colonies which were always Tet^r. In contrast, after preservation in glycerol at −70°C, 30% of the surviving cells were Lac$^-$ and Tra$^-$ (i.e., resistant to R17 at 34°C), although they were still Tet^r. This could indicate a high sensitivity of Hfrq to freezing conditions.

Chromosomal gene transfer by Hfrq. We compared the frequencies of transfer of chromosomal markers according to mating conditions with crosses between Hfrq and AK372 (*his-1*); the frequency of His$^+$ transfer was 2.0×10^{-6} (recombinants per input donor cell) when mating was carried out for 3 h at 30°C in liquid medium and 8.4×10^{-5} when mating was carried out at 37°C on membrane, as described above. Therefore, the best conditions were achieved when mating was performed at 37°C on membrane. At this temperature all markers tested were transferred in 2 h (except *met-8*; Table 4), whereas at least 3 h were necessary at 30°C.

In mating with Hfrq, a sharp gradient of transfer was observed; some recombinants (Met$^+$, Xyl$^+$, Arg$^+$, Ile$^+$) appeared at a very high frequency, i.e., higher than 10^{-1}, whereas others (Ade$^+$, His$^+$) appeared at a frequency lower than 10^{-4}. For each selected marker, 100 recombinants were picked up on selection plates and gridded on the same medium. The number of recombinants which had acquired unselected markers from the donor strain was scored by replica plating (Table 4). Strong linkage ap-

TABLE 4. Transfer of chromosomal markers from Hfrq and linkage between markers[a]

Recipient strain[b]	Selected character	Transfer frequency[c]	Unselected character	Cotransfer frequency[d]
AK3710 (*arg-1 ile-1*)	Arg^+	1.1×10^{-1}	Ile^+	54
	Ile^+	1.0×10^{-1}	Arg^+	67
AK3711 (*arg-1 met-3*)	Arg^+	1.2×10^{-1}	Met^+	28
	Met^+	9.9×10^{-1}	Arg^+	4
AK3720 (*his-1 gal-1*)	His^+	1.8×10^{-4}	Gal^+	1
	Gal^+	5.8×10^{-4}	His^+	23
	Tet^r	1.1×10^{-3}	His^+	0
			Gal^+	0
			Lac^+	100
AK3727 (*his-1 trp-1*)	His^+	8.6×10^{-5}	Trp^+	57
			Nal^r	1
			Tet^r	0
	Trp^+	2.1×10^{-4}	His^+	20
			Nal^r	0
			Tet^r	0
	Tet^r	5.6×10^{-4}	His^+	0
			Trp^+	0
			Nal^r	1
AK3731 (*leu-1 ile-4*)	Leu^+	4.9×10^{-2}	Ile^+	14
	Ile^+	2.5×10^{-1}	Leu^+	8
AK3733 (*leu-1 his-2*)	Leu^+	6.0×10^{-2}	His^+	0
	His^+	1.2×10^{-4}	Leu^+	19
AK3812 (*leu-3 met-1*)	Leu^+	4.5×10^{-2}	Met^+	23
	Met^+	9.7×10^{-1}	Leu^+	3
AK3841 (*xyl-1 met-7*)	Met^+	1.4×10^{0}	Xyl^+	24
	Xyl^+	7.6×10^{-1}	Met^+	37
AK37163 (*arg-1 leu-1 cys-4*)	Arg^+	1.3×10^{-1}	Leu^+	7
			Cys^+	7
	Leu^+	6.5×10^{-2}	Arg^+	25
			Cys^+	31
	Cys^+	8.4×10^{-2}	Arg^+	20
			Leu^+	25
AK37164 (*arg-1 leu-1 met-8*)	Arg^+	1.0×10^{-1}	Leu^+	15
			Met^+	0
	Leu^+	5.9×10^{-2}	Arg^+	17
			Met^+	0
	Met^+	$<10^{-8}$		
AK37166 (*arg-1 leu-1 ura-1*)	Arg^+	1.1×10^{-1}	Leu^+	9
			Ura^+	0
	Ura^+	6.1×10^{-3}	Arg^+	26
			Leu^+	20
AK37345 (*leu-1 thr-3 pur-3*)	Leu^+	5.5×10^{-2}	Thr^+	54
			Ade^+	0
	Thr^+	5.5×10^{-2}	Leu^+	64
			Ade^+	0
	Ade^+	4.7×10^{-5}	Leu^+	0
			Thr^+	1
	Tet^r	6.3×10^{-4}	Leu^+	0
			Ade^+	0
			Lac^+	100
AK38454 (*xyl-1 leu-1 pan-2*)	Xyl^+	1.7×10^{-1}	Leu^+	3
			Pan^+	2
	Leu^+	6.0×10^{-2}	Xyl^+	20
			Pan^+	25
	Pan^+	2.8×10^{-2}	Xyl^+	18
			Leu^+	30

[a] Matings were done on membranes for 2 h at 37°C as described in the text. The donor was counterselected by streptomycin in the selection plates.

[b] The recipient strains are presented with their relevant genotype, and they were Str^r. The Ile^- mutants were unable to grow without isoleucine, and the presence of the marker *pur-3* was scored as an auxotrophic requirement for adenine.

[c] The transfer frequency is the number of recombinants for the selected character per input donor cell of Hfrq.

[d] The cotransfer frequency is the percentage of recombinants which has acquired the unselected marker with the selected one (100 colonies tested).

peared between *leu* and *thr*, *arg* and *ile*, *his* and *trp*, and *met* and *xyl*. For two given genes, an asymmetry always existed in the cotransfer frequency, depending on whether one or the other marker was selected. Each tested marker showed some linkage with the others, except Tet^r and Lac^+, which were 100% linked between themselves (500 colonies tested) but were not linked to other markers (data not shown).

No genetical transfer was observed from the strain 3937jN, whereas 3937jN(F') could transfer Leu^+, but at a frequency 1,000 times lower than that obtained with strain Hfrq.

With the technique of Chatterjee and Starr (8; see above), it was possible to measure the kinetics of chromosomal transfer. The results shown in Fig. 1 emphasized a different time of entry for each marker; with Hfrq as the donor strain, the order of entry in the recipient cell was $Met^+ \ldots Xyl^+ \ldots Ile^+ \ldots Leu^+ \ldots Cys^+ \ldots$. The behavior of Tet^r was different from the others; although its time of entry was rather early (less than 1 h), its transfer frequency stayed low and did not reach 10^{-5} Tet^r cells per recipient cell after 3 h of mating at 30°C (Fig. 1).

Transfer of auxotrophic markers. To check whether the genes introduced into *E. carotovora* subsp. *chrysanthemi* by our transfer system were incorporated by recombination in the chromosome of the recipient cell, we tried to transfer recessive phenotypes. As auxotrophies are usually recessive, we constructed auxotrophic donor strains by transferring the F'(Ts)::Tn*10* Lac^+ episome in strains having two auxotrophies and selecting Hfrs in the same way as it was done for Hfrq. These double-mutant Hfr strains were mated with recipients carrying two auxotrophic mutations different from those present in the donor. Transconjugants prototrophic for one of the phenotypes mutated in the recipients were selected and screened for the presence of auxotrophic markers received from the donor (Table 5). This way, Leu^-, Ile^-, and His^- were transferred from the donor strains H311 and H331.

DISCUSSION

The results of our study demonstrated that it was possible to transfer an F' episome from *E. coli* to *E. carotovora* subsp. *chrysanthemi*, although all of the strains of this species were not able to receive and express the fertility functions. This finding corroborates the positive results obtained by Chatterjee and Starr (7) and by Prokulevich and Fomichev (24) and could explain the negative results described by others

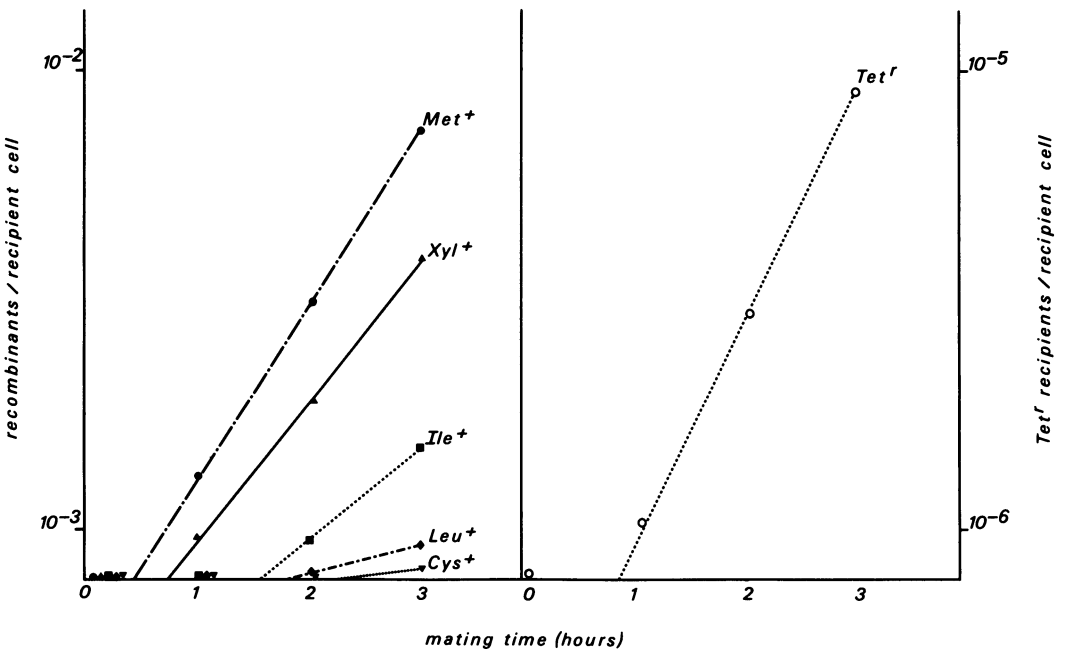

FIG. 1. Kinetics of transfer of chromosomal markers from *E. carotovora* subsp. *chrysanthemi* donor strain Hfrq to several auxotrophic recipient strains. Transfer of Met^+ (●) and Tet^r (○) was tested with AK3812 (*leu-3*, *met-1*), transfer of Leu^+ (◆) and Cys^+ (▼) was tested with AK37163 (*arg-1*, *leu-1*, *cys-4*), and the transfer of Xyl^+ (▲) and Ile^+ (■) was tested with AK38452 (*xyl-1*, *leu-1*, *ile-6*). Interrupted matings were carried out at 30°C as described in the text. The Ile^- mutants were isolated as unable to grow without isoleucine.

TABLE 5. Inheritance of auxotrophic markers transferred by auxotrophic donor strains of *E. carotovora* subsp. *chrysanthemi*

Cross[a]	Selected phenotype	Unselected phenotype[b]	Cotransfer frequency[c]
H311 (*leu-1 ile-4*) × AK3712 (*arg-1 thr-2*)	Thr^+	Leu^-	62
		$Leu^- Arg^+$	9
	Arg^+	Leu^-	11
		$Leu^- Thr^+$	7
		Ile^-	69
		$Ile^- Thr^+$	13
H331 (*leu-1 his-2*) × AK3712 (*arg-1 thr-2*)	Thr^+	Leu^-	58
		$Leu^- Arg^+$	34
	Arg^+	Leu^-	14
		$Leu^- Thr^+$	13
H311 (*leu-1 ile-4*) × AK3842 (*xyl-1 trp-3*)	Trp^+	Leu^-	17
		$Leu^- Xyl^+$	2
H331 (*leu-1 his-2*) × AK3842 (*xyl-1 trp-3*)	Trp^+	Leu^-	9
		$Leu^- Xyl^+$	6
		His^-	16

[a] Matings were performed on membranes for 2 h at 37°C as described in the text. Strains H311 and H331 were auxotrophic and Str^r Hfr donors. Donors were counterselected by absence of a growth factor in the selection plates.

[b] Presence of an auxotrophic phenotype of the donor was detected in the recombinants by replica plating.

[c] The frequency of cotransfer is defined in footnote *d* of Table 4.

(11, 15; W. V. Guimaraes, Ph.D. thesis, University of California, Berkeley, 1976).

There are several explanations for the observation that F'(Ts)::Tn*10* Lac^+ was not easily transferred to strain 3920 (Table 2). One is that this strain would contain a plasmid of the same incompatibility group. However, a preliminary search for plasmid(s) in 3920 by the technique of Casse et al. (5) remained unsuccessful. The absence of transfer could also be due to the presence in 3920 of very active restriction enzyme(s).

On the other hand, 3920 is also a poor donor of F'(Ts)::Tn*10* Lac^+ (Table 3). It is known that in *E. coli* at least four chromosomal genes are necessary for F transfer (3, 21, 27). It is possible that equivalent genes are either absent or inactive in strain 3920.

As previously reported (7, 11, 15, 24), the F' factor was very unstable in the strain 3937jN(F'). The functions encoded by the episome F'(Ts)::Tn*10* Lac^+ were expressed in this strain, since it was Lac^+, Tet^r, and Tra^+. Furthermore, the episome remained thermosensitive for its replication at 40°C; this allowed us to make use of the thermosensitivity in *E. carotovora* subsp. *chrysanthemi*, whose maximum growth temperature is about 41.5°C (12). Our results have shown that we succeeded in selection of clones which transferred genes at high frequencies. Thermosensitivity of the plasmid Rts1 (15; Guimaraes, Ph.D. thesis) has already been used in *E. carotovora* subsp. *chrysanthemi*, but this was the first time that this property was successfully used with a F' episome in *Erwinia* (9; Guimaraes, Ph.D. thesis).

The most likely hypothesis is that at 40°C in presence of tetracycline, we selected clones by integrative suppression of the thermosensitivity of the episome (2, 23). Although we have no molecular evidence that the strain Hfrq was a true Hfr, the following genetic evidence exists.

(i) The phenotypes controlled by the episome were stable in Hfrq, but not in the parental strain 3937jN(F').

(ii) When crossed with auxotrophic recipient strains, every auxotrophic marker tested (except *met-8*, which may be a double mutation) was transferred by Hfrq (Table 4).

(iii) Hfrq transferred the Leu^+ chromosomal marker at a frequency 1,000 times higher than that obtained with the F' strain; this is in good agreement with the difference observed between the chromosomal transfer frequency by F' donor strain and by Hfr in *E. coli* (28). Furthermore, the transfer frequencies observed (close to 10^0 for Met^-; Table 4) were about the same magnitude as those obtained with Hfr donors of *E. coli* (29).

(iv) Chromosomal transfer from Hfrq was polarized. Three series of data established this fact. The first was the existence of a transfer gradient; some genes were transferred at a frequency higher than 10^{-1} recombinant per Hfrq donor cell, whereas others were transferred at a frequency of less than 10^{-4} recombinant per donor cell (Table 4). The second was the asymmetry always observed in the frequencies of

cotransfer of two given genes (Table 4). The third was the existence of different times of entry for the genetic markers, as evidenced by interrupted mating experiments (Fig. 1). Thus, the genetic transfer from Hfrq would proceed from a fixed and unique origin on its chromosome and in one determined direction. However, the cotransfer frequencies obtained when *pur-3* was the selected marker could indicate a transfer at low frequency in the opposite direction. A similar situation was observed with the same episome in *S. typhimurium* (10).

(v) It was possible to construct new auxotrophic strains by transferring auxotrophic markers (i.e., supposed to be recessive; Table 5); we can thus reasonably suppose that the genetic material, which enters into the recipient cell during the mating with an Hfrq donor cell, is incorporated into the chromosome by homologous recombination.

Being carried by the episome, Tet^r should be transferred by the Hfr cells as a very late marker, thus at a frequency much lower than 10^{-4}. But in Hfrq, it was not possible to show a linkage between the markers borne by the episome (Lac^+, Tet^r, Tra^+) and the chromosomal markers (Table 4 and above). Furthermore, the transfer frequency of Tet^r was higher than those of His^+ and Ade^+, and its time of entry seemed to be less than 1 h (Fig. 1). Together, these data could be interpreted as follows: in the growing population of Hfrq, the episome would return to the autonomous state by excision, and a small proportion of donor cells could have become F′ at the time of the mating; the transfer of Tet^r by these F′ cells would be more frequent and would hide that due to the Hfr cells.

With our system, it becomes possible to build new strains (Table 5) and to map the chromosome of *E. carotovora* subsp. *chrysanthemi* 3937j. The data of the three mapping methods (transfer gradient, measurement of linkage by the frequencies of cotransfer, and interrupted matings) have allowed us to establish the most probable sequence for the chromosomal genes studied. The strain Hfrq transferred its chromosome in the following order: origin. . .*met*. . . *xyl*. . .*arg*. . .*ile*. . .*leu*. . .*thr*. . .*(cys)*. . .*pan* . . .*(ura)*. . .*(gal)*. . .*trp*. . .*his*. . .*pur*. . . (Fig. 2), with the following uncertainties.

(i) According to the gradient of transfer and to the cotransfer frequencies, *cys* would map before *leu* (Table 4); but *cys* entered the recipients after *leu* (Fig. 1).

(ii) The map position of *ura* is not well defined between *pan* and *gal*, because the linkage of these markers with *ura* was not measured.

(iii) According to the frequencies of cotransfer, *his* should map before *gal*, although the opposite order is suggested by the gradient of transfer (Table 4). We consider that the results obtained from the gradient of transfer are usually more reliable than those given by cotransfer frequencies.

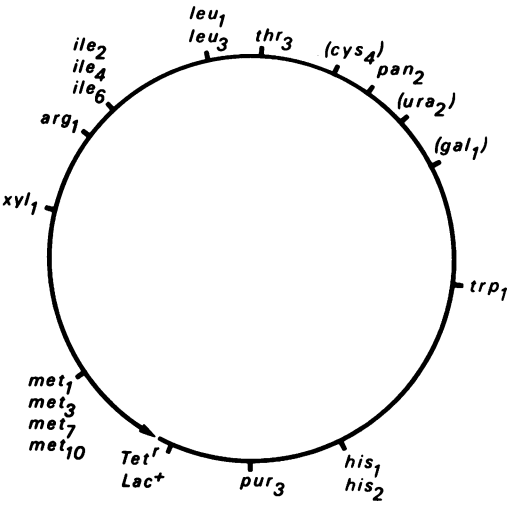

FIG. 2. Linkage map of *E. carotovora* subsp. *chrysanthemi* 3937j with origin and direction of transfer from Hfrq. The position of the markers put between brackets is not determined without ambiguity (see text).

This sequence allowed us to infer that the genetic map of the chromosome of *E. carotovora* subsp. *chrysanthemi* 3937j would be roughly like that of *E. coli* K-12 (1), but with an inversion of the *thr*. . .*leu* region and maybe of the *arg*. . .*ile* region too (84 to 89 min on the map of *E. coli* [1]). If the map of *E. carotovora* subsp. *chrysanthemi* is referenced by analogy with that of *E. coli*, the transfer origin of Hfrq would be located in the region included between min 50 and 62, and the transfer would occur in the clockwise direction (Fig. 2). Together, these results fairly confirm those obtained with *E. carotovora* subsp. *chrysanthemi* EC16 (6, 7), but with the following differences.

(i) Strain 3937j expressed recipient and donor abilities for the F′ factor clearly greater than those of strain EC16 (7), and it stems from this that the transfer frequencies for the chromosomal genes were 1,000 times higher with the strain Hfrq compared with Hfr8 (which derives from EC16; 8). This difference may also be due to the mating temperature which we chose: 37°C with Hfrq as donor strain, instead of 30°C with Hfr8.

(ii) Overall, the origin and direction of transfer from Hfrq would very probably be different from those of Hfr8, which is stemming from an integration of the episome F′Lac^+ at the site of EC16 *lac* genes (8) (*E. carotovora* subsp. *chrysanthemi*, although phenotypically Lac^-, has a

gene coding for a β-galactosidase). The donor strain Hfr8 transfers *leu* and *thr* as the earliest markers (6, 8). Unless the *lac* gene(s) of *E. carotovora* subsp. *chrysanthemi* 3937j is located at a very different place from that of *E. coli*, it seems that the integration of the F'(Ts)::Tn*10* Lac^+ factor, which has been at the origin of the strain Hfrq, would not have been the consequence of the homology between two *lac* genes, one on the chromosome and the other on the episome. One alternative possibility could be that the Tn*10* promoted the formation of a cointegrate structure between the chromosome and the episome (26). This cointegrate would be stabilized for unknown reasons.

If this hypothesis were right, it would be possible to isolate in *E. carotovora* subsp. *chrysanthemi* other Hfr strains with various origins and directions of transfer, which would allow transfer of any part of the chromosome at high frequency. Encouraging preliminary results have been obtained in that direction. By taking advantage of the imprecise excision of the episome, it should also be possible to isolate F' strains which carry various parts of the *Erwinia* chromosome.

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