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Transposon-Facilitated Recombination in Classical Biotypes of *Vibrio cholerae*

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Transposon-facilitated recombination (Tfr) donors of classical *Vibrio cholerae* strain 162 were constructed by introducing the ampicillin transposon Tn*1* into the P conjugative plasmid and the bacterial chromosome. The improved donors mediated high-frequency, polarized transfer of chromosomal genes from origins specified by the chromosomal Tn*1* insertion site. Classical Tfr donors were used to confirm the gene orders of the previous classical strain 162 genetic map and to establish its circularity. Significant transfer of linked genes from El Tor Tfr donors to classical recipients was demonstrated, and other evidence for genetic relatedness of these two *V. cholerae* biotypes is discussed.

El Tor and classical biotypes are the most important of the several biotypes of *Vibrio cholerae* capable of causing human cholera. Previous cholera pandemics resulted from the spread of classical vibrios, but since about 1960, El Tor biotypes have largely supplanted classical strains as the causative agent of epidemic cholera (7). El Tor vibrios differ from classical biotypes in only a few characteristics, and taxonomic studies have indicated that these two groups are probably members of the same species (4, 6). Although gene transfer by conjugation is well documented in *V. cholerae* (1, 2, 11, 20), the degree of relatedness of these two biotypes has remained unclear because genetic comparisons between them have been limited.

Conjugal gene transfer in *V. cholerae* is mediated by a naturally occurring conjugative plasmid called P (2). The *V. cholerae* mating system is similar to F-mediated conjugation in *Escherichia coli* except that gene transfer is less efficient, and P^+ *V. cholerae* are apparently incapable of forming Hfr donors (20). Parker and coworkers constructed a relatively coherent linear map of the classical strain 162 chromosome by measuring linkages between selected and unselected markers in $P^+ \times P^-$ crosses (20; Fig. 1). However, this mapping procedure is inefficient because transfer frequencies are low (10^{-5} to 10^{-6} per input donor) and markers are poorly linked.

A more efficient mating system that used improved "transposon-facilitated recombination" (Tfr) donors of El Tor strain RJ1 was recently described (11). Tfr donors contained identical copies of the ampicillin transposon Tn*1* (8, 12) in the P plasmid and bacterial chromosome to provide homology for P-factor integration. The improved donors initiated high-frequency, polarized transfer from origins specified by the chromosomal Tn*1* insertion site. Direction of gene transfer from a given donor could be reversed by using as sex factor either of two P:: Tn*1* conjugative plasmids that appeared to contain oppositely oriented Tn*1* insertions. The circular genetic map of the El Tor strain obtained with Tfr donors resembled in most respects the previously obtained linear map of classical strain 162 (S. R. Johnson, 1978, University of California, Los Angeles, 1978).

In this study, procedures developed for obtaining El Tor Tfr donors were modified to obtain similar Tfr donors in the classical strain 162. By using classical Tfr donors, most of the gene orders of the classical strain 162 map were confirmed, and significant linkage was detected between the previously unlinked *pro-2* and *his-1* markers that comprised its termini. Similarities between the linkage maps of El Tor and classical strains are discussed and preliminary results of interstrain crosses between these two biotypes are presented.

MATERIALS AND METHODS

Bacterial strains and plasmids. *V. cholerae* strains and plasmids are listed in Table 1. Recipients were either identical to, or were derived from, the classical strain RV33 mutants of Parker et al. (20), and donors were constructed in a thymine-requiring (*thy-20*) mutant of their strain RV34. Antibiotic resistance mutations for counterselection were introduced into recipients as required. El Tor strains and P-plasmid derivatives were provided by S. Johnson (11). Bacteria were maintained at −70°C in brain heart infusion broth (Difco) containing 15% (vol/vol) glycerol.

Media. Meat extract agar supplemented with 0.2% yeast extract was used as nutrient solid medium (19). Brain heart infusion broth with 0.2% yeast extract was used in mating experiments. The solid synthetic me-

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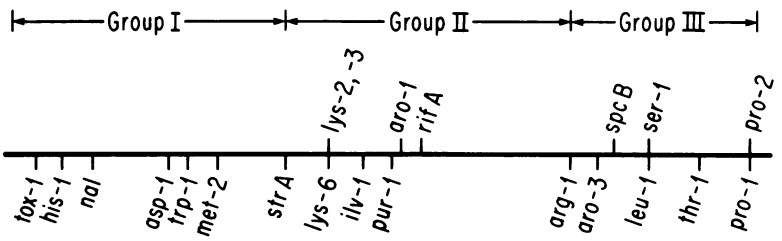

FIG. 1. *V. cholerae strain 162 linkage map with P⁺ donors (redrawn from Parker et al., 20). Markers below the horizontal line were ordered; markers above the line were linked, but were not ordered with respect to other markers. Map distances correspond approximately to genetic linkages. Group I, II, and III markers have been defined (20).*

TABLE 1. *V. cholerae strains and plasmids*

Strain 162	Genotype[a]/relevant properties	Derivation/reference
Recipients		
RV33	*arg-1 ilv-1 his-1*	From classical strain 162 (2, 20)
RV86	*arg-1 ilv-1 his-1 trp-1*	From RV33 (20)
RV151	*arg-1 ilv-1 his-1 trp-1 ura-6*	From RV86 (20)
RV169	*arg-1 ilv-1 his-1 trp-1 met-2 rif*	From RV86 (20)
RV175	*arg-1 ilv-1 his-1 trp-1 lys-6 rif*	From RV86 (20)
RV183	*arg-1 ilv-1 his-1 trp-1 pro-2*	From RV86 (20)
RV197	*arg-1 ilv-1 his-1 trp-1 asp-1*	From RV86 (20)
RV202	*arg-1 ilv-1 his-1 trp-1 cys-5*	From RV86 (20)
RV307	*arg-1 ilv-1 his-1 trp-1 met-2 cys-11 spc*	From RV169, this study
RV309	*arg-1 ilv-1 his-1 trp-1 pro-2 aro-11*	From RV183, this study
RV301	*arg-1 ilv-1 his-1 aro-10*	From RV33, this study
RV302	*arg-1 ilv-1 his-1 lys-10 strA*	From RV33, this study
RV303	*arg-1 ilv-1 his-1 ser-10*	From RV33, this study
RV308	*arg-1 ilv-1 his-1 cys-10*	From RV33, this study
Donors		
RV34	*leu-1 pur-1*	From classical strain 162 (2, 20)
RV312	*leu-1 pur-1 thy-20*	From RV34, this study
RV340	*leu-1 pur-1 thy-20 ilv10*::Tn*1*	From RV312, this study
RV343	*leu-1 pur-1 thy-20 met-10*::Tn*1*	From RV312, this study
RV346	*leu-1 pur-1 thy-20 arg-10*::Tn*1*	From RV312, this study
El Tor donor		
RJ233	Prototroph, Ap^r, Tn*1* insertion between *str* and *met-2*	(11)
Plasmids		
P	Naturally occurring *V. cholerae* conjugative plasmid	(2)
pSJ5	P::Tn*1* hybrid, Tn*1* in (+) orientation, Ap^r	(11, 12)
pSJ13	P::Tn*1* hybrid, Tn*1* in (−) orientation, Ap^r	(11)
pSJ25	P::Tn*9* hybrid, contains *hts* mutation that renders *h*ost *t*emperature *s*ensitive, CM^r	(11)
pSJ26	pSJ25::Tn*1*, contains *hts* mutation, Cm^r, Ap^r	(11)

[a] Designations correspond to those of Parker et al. (20) and Johnson and Romig (11) for *V. cholerae*. The antibiotic resistance mutations listed were used for linkage measurements.

dium contained Difco minimal broth Davis, 2 g of glucose, and 15 g of agar per liter and was supplemented with 50 μg of the required amino acids per ml. Antibiotics were used at the following concentrations: 100 μg of streptomycin, 100 μg of spectinomycin, 400 μg of penicillin G, and 30 μg of chloramphenicol per ml. Rifampicin and nalidixic acid were used at 30 and 50 μg/ml, respectively, in meat extract agar; in synthetic media, both were used at 15 μg/ml.

Mutant isolation. "Standard" auxotrophic mutants were isolated by mutagenesis with *N*-methyl-*N'*-nitro-nitrosoguanidine (NTG) as previously described (20). Spontaneous antibiotic-resistant mutants were selected by plating about 10^9 bacteria on meat extract agar plates containing appropriate antibiotics. Spontaneous thymine auxotrophs were selected with trimethoprim (17).

Construction of Tfr donors. The methods of Johnson and Romig (11) were used to construct strains that contained chromosomally inserted copies of the ampicillin transposon Tn*1*. Briefly, the thermosensitive hybrid P plasmid, pSJ26, was transferred to strain

RV312, and transconjugants were selected with penicillin and chloramphenicol. The transconjugants were incubated at 42°C to isolate severely deleted, transfer-deficient mutants of plasmid pSJ26 to serve as Tn*1* vectors. Survivors that retained penicillin resistance and simultaneously lost Cm^r and the *hts-25* phenotype, contained plasmid P::Tn*1* deletion mutants. The resulting strain was grown overnight to allow Tn*1* to transpose and then was superinfected with the incompatible, thermosensitive PSJ25 plasmid to eliminate the Tn*1* vector. Transconjugants were selected with penicillin and chloramphenicol at 25°C to obtain strains that contained Tn*1* transpositions.

Unlike El Tor strain RJ1 that contained no detectable plasmids, classical strain 162 contains two cryptic plasmids (5, 12). Because Tn*1* has been shown to transpose preferentially to plasmids (14), additional procedures were required to identify transposition strains that contained the transposon in their chromosome. Accordingly, after eliminating the thermosensitive pSJ25 plasmid, Tn*1* transposition strains were enriched by nalidixic acid (10) or thymineless death (15) to obtain auxotrophic mutants that resulted from Tn*1* insertions into biosynthetic genes. The auxotrophic mutants were converted into Tfr donors with conjugative plasmids pSJ5(+) and pSJ13(−). Properties of classical Tfr donors are described in Results.

Mating procedures. Conjugal transfer mediated by P::Tn*1* sex factors in Tn*1* chromosomal insertion strains (Tfr conjugation) was performed as previously described (11). Logarithmically growing donor and recipient cultures were mixed at a ratio of 1:10 in brain heart infusion broth and shaken at 100 rpm at 37°C for 150 min. Dilutions of the mating mixture were spread on appropriate synthetic or other selective media, and the donor strain was usually counterselected with an antibiotic. To avoid counterselective biases, markers used for counterselection were located as distally as possible on the donor chromosome. For linkage analyses, single donor alleles were selected on media supplemented with the other nutritional requirements of the donor and recipient, and recombinants were scored for unselected markers by replica plating.

Beta-lactamase assay. Beta-lactamase was assayed by the hydrolysis of the chromogenic cephalosporin 87/312 (Glaxo Group Research Ltd., Middlesex, England) as described by O'Callaghan et al. (18).

RESULTS

By using the described procedures, presumed Tn*1* insertion auxotrophs that required either isoleucine-valine, methionine, or arginine were obtained. To determine whether the auxotrophic mutations resulted from Tn*1* insertions in the relevant genes, spontaneous and NTG-induced reversion frequencies at these loci were measured. Consistent with results obtained with other insertion mutations (22), the reversion frequencies of these mutations were not enhanced with NTG, whereas reversions at other loci were enhanced several hundred-fold (data not shown). The Tn*1* insertion auxotrophs reverted spontaneously at frequencies of less than 10^{-8} except for the *arg-10*::Tn*1* mutation in strain RV346, which spontaneously reverted at a frequency of from 1×10^{-6} to 3×10^{-6}. Because Tn*1* excisions rarely restore gene function, it seemed likely that this Tn*1* insertion was located outside the gene boundaries and interferred with its expression by polarity (23).

Determining the origins and directions of transfer of classical Tfr donors was facilitated by assuming that their Tn*1* insertion sites were in, or near, previously mapped standard auxotrophic mutations that exhibited the same phenotype. Tranfer frequencies (Table 2) of genes near these locations (Fig. 1) indicated that the classical Tfr donors mediated polarized gene tranfer and, except for strain RV340, that their direction of chromosomal transfer was reversed when the alternative conjugative plasmid was used. The lack of ability to reverse direction of chromosomal transfer in Tfr donor RV340 was unexpected because this ability was found in previously examined transposon-facilitated donors (3, 11, 13).

Further examination of strain RV340(pSJ5) showed that although either the *ilv-1* or *arg-1* marker was transferred at high frequency as a selected marker, neither of these genes was cotranferred as an unselected marker when the

TABLE 2. *Transfer frequencies*[a] *of selected markers by classical Tfr donors*

Donor strain	Conjugative plasmid	Frequency of recombinants for selected markers[b]				
		ilv-1	*arg-1*	*pro-2*	*his-1*	*trp-1*
RV340 (*ilv-10*::Tn*1*)	pSJ5(+)	100	23		10	1
	pSJ13(−)	70	27		10	3
RV343 (*met-10*::Tn*1*)	pSJ5(+)	3	3		35	100
	pSJ13(−)	100	45		4	3
RV346 (*arg-10*::Tn*1*)	pSJ5 (+)	<1		1,000	20	40
	pSJ13(−)	320		<1	0	<1

[a] Recombinants per 10^5 input donors.

[b] RV169 *spc* was the recipient for Tfr donors RV340 and RV343; RV183 *str* was the recipient Trf donor RV346.

other gene was selected (less than 1% co-transfer, Table 3). The lack of the ability to co-transfer these linked genes indicated that the transfer origin in the *ilv-10*::Tn*1* mutation of strain RV340 was located between the *ilv-1* and *arg-1* genes. The high frequency transfer of both markers implied that either P::Tn*1* conjugative plasmid could promote chromosomal transfer in both directions from the Tn*1* origin. This latter possibility seemed unlikely because recombination between homologous Tn*1* sequences, required to integrate a given P::Tn*1* plasmid, should be possible in only one orientation.

The apparent contradiction was resolved by assuming that strain RV340 contained two chromosomal copies of Tn*1* inserted in reverse orientation between the *ilv-1* and *arg-1* markers. In this arrangement, either P::Tn*1* conjugative plasmid could integrate with equal probability into either of the oppositely oriented chromosomal Tn*1* insertions in a given cell. In this case, donor populations of strain RV340 would consist of two classes of Tfr donors: one that transferred *ilv-1* as a high-frequency proximal marker and *arg-1* as an unlinked terminal marker, and another class that transferred *arg-1* as a proximal marker and *ilv-1* as a low-frequency terminal marker (Fig. 2). In addition to its effects on transfer frequencies and linkages, this interpretation predicted that strain RV340 should contain higher concentrations of TEM beta-lactamase (9) than Tn*1* insertion strains that contained a single copy of the ampicillin transposon. Results of beta-lactamase assays to test this prediction showed that strain RV340 contained about twofold more enzyme activity than strain RV346 and thus were consistent with the above interpretation (Fig. 3).

TABLE 3. *Effects of the selected marker on transfer of linked genes by Tfr donor RV340*

Tfr donor[a]	Marker tested (% co-transfer)[b]				
	strA	*lys-10*	*ilv-1*	*arg-1*	*leu-1*
RV340(pSJ5)	63	65	Select	<1	<1
	<1	<1	<1	Select	12
Control: RV343(pSJ13)		10	38	Select	8

[a] Donors were counterselected with nalidixic acid.
[b] About 200 recombinants were tested.

Gene transfer by classical Tfr donors. To further assess transfer properties, linkages for the group III markers of Parker et al. (20) obtained with Tfr donor RV340 were compared with the linkages obtained with P^+ donors. The gene orders, which were determined by measuring linkages of unselected markers to the selected *arg-1* marker, agreed well in both kinds

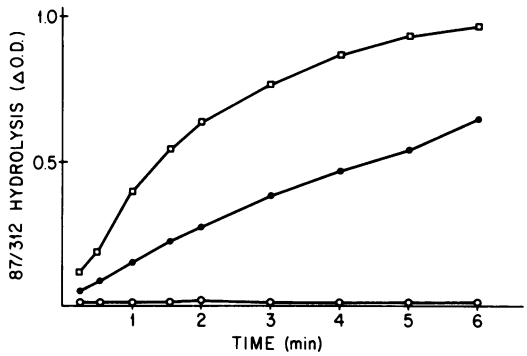

FIG. 3. *Rate of hydrolysis of the chromogenic cephalosporin 87/312 (Glaxo) by V. cholerae strains. Equal concentrations of logarithmically growing cultures were centrifuged, resuspended in 20% sucrose, and osmotically lysed by rapid dilution. Beta-lactamase was assayed in reaction mixtures containing 0.7 ml of phosphate buffer (5 mM, pH 7.0), 0.1 ml of a 10^{-3} M solution of cephalosporin, and 0.3 ml of bacterial lysate in a 1-cm cuvette. The change in absorbance at 482 nm was measured in a Beckman spectrophotometer at the indicated times. Symbols: □, Strain RV340; ●, strain RV346; ○, ampicillin-sensitive control strain RV312.*

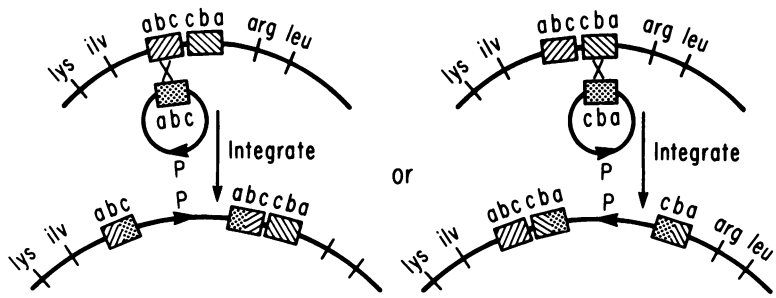

FIG. 2. *Model for the formation of two classes of Tfr donors in populations of strain RV340. Rectangular boxes represent Tn1 insertions and are lettered to indicate relative orientations. The arrow represents the transfer origin of the P::Tn1 conjugative plasmid.*

of crosses, although linkage values obtained with the Tfr donor were higher (Table 4). The linkage between two markers, such as *arg-1* and *ser-10*, was higher when the distally transferred marker, *ser-10*, was selected (Table 5) than when the proximally transferred marker, *arg-1*, was selected (Table 4). These polarized transfer effects were not seen with P^+ donors. Linkages of the various selected markers to the unselected donor *leu-1* marker were also generally consistent with previously assigned gene orders (Table 5). The close linkage of the *cys* mutations to *arg-1*, which was not detected with P^+ donors, was abolished when donors were counterselected with spectinomycin.

Linkages of other markers obtained with Tfr donors were likewise generally consistent with linkages obtained in $P^+ \times P^-$ crosses (Tables 6 and 7). A notable exception was the significant co-transfer of the *pro-2* and *his-1* markers by Tfr donor RV343(pSJ5). The failure to detect linkage between the *his-1* and *pro-2* markers in $P^+ \times P^-$ crosses resulted in a linear map that contained these two markers at its termini. The ability to link these terminal markers with Tfr donors, together with previous mapping data (20), strongly suggested that the *V. cholerae* chromosome is circular. The linkage observed with other tested markers supported this suggestion.

Gene transfer from El Tor donors to classical recipients. The least satisfactory linkages obtained with classical Tfr donors were in the *met-his-trp* region, particularly in the crosses used to detect linkage of these genes to the *str-rif-ilv* markers. The availability of El Tor Tfr donor RJ223(pSJ13), which transferred *met-2* as a proximal marker, prompted us to investigate its usefulness for measuring linkages in this region.

The El Tor Tfr donor was crossed to the classical recipient RV169, and the co-transfer of unselected markers in recombinants individually selected for the donor met^+, trp^+, or his^+ genes was determined. The results (Table 8) indicated that El Tor markers were efficiently transferred to the classical recipient. Higher linkages were obtained in this cross than in the corresponding homologous crosses, and polarity effects were consistent with the assigned gene order. In similar experiments, other El Tor donors were shown to transfer markers at about the same frequencies either to classical or to El Tor recipients. However, transfer frequencies from classical donors were generally at least 10-fold lower to El Tor recipients than to classical recipients. The reason for this asymmetric transfer is not known.

DISCUSSION

Tfr donors of classical *V. cholerae* strain 162 were constructed by isolating auxotrophic mutants that resulted from Tn*1* insertions into biosynthetic genes. This isolation procedure was advantageous because it did not enrich for Tn*1* insertions in the cryptic plasmids of the classical strains. However, because Tn*1* chromosomal insertions are rare, and most of them apparently do not cause auxotrophic mutations (11), only a limited number of Tn*1* insertion auxotrophs were obtained.

Locating chromosomal Tn*1* insertion sites, and hence transfer origins, of these strains was facilitated by assuming that the resulting auxotrophic mutations were in or near previously mapped, phenotypically similar standard mutations. This assumption was validated by showing that classical Tfr donors transferred genes prox-

TABLE 5. *Linkage of the unselected arg-1 and leu-1 markers to various selected markers with Tfr donor RV340*[a]

Selected markers	Counterselective markers	Co-transfer with selected marker (%)	
		arg-1	*leu-1*
cys-5	*thy-20, pur-1*	73	22
cys-11	*nal*	48	
cys-10	*rif, thy-20, pur-1*	62	
ura-6	*str*	49	33
ser-10	*nal*	54	50
pro-2	*rif*	14	33

[a] The recipients were the same as those used in Table 4.

TABLE 4. *Linkage of various unselected markers to the selected arg-1 marker with Tfr donor RV340*[a]

Donor	Marker tested (% co-transfer)									
	ilv-1	*arg-1*	*spc*	*cys-5*	*cys-10*	*cys-11*	*ura-6*	*ser-10*	*leu-1*	*pro-2*
RV340	(0.3)[b]	Select Select	27 (16)	(<1)	57	34	(<1)	19 (6)	12 (3)	4 (1)

[a] All recipients contained the *arg-1* and *ilv-1* mutations; other mutations were in the various strains listed in Table 1.

[b] Co-transfer values for P^+ donors from Parker et al. (20). The *cys-10, -11* and *ser-10* mutations were isolated in this study.

TABLE 6. *Linkage of group I and II markers with Tfr donor*[a]

Donor	Markers tested (% co-transfer)							Counter-select
	met-2	*thy-20*	*strA*	*rif*	*lys-6*	*ilv-1*	*aro-11*	
RV340(pSJ5)	Select			2		0		*spc*
		2		59	Select	81		*spc*
		7	63		65[b]	Select		*nal*
						65	Select	*str*

[a] Group I, II, and III markers were defined by Parker et al. (20).
[b] This value is for the *lys-10* mutation in strain RV302 and is similar to values for the *lys-6* mutation.

TABLE 7. *Linkage of markers in group I and III with Tfr donors*[a]

Tfr donor	Marker tested (% co-transfer)							Counter-select
	leu-1	*pro-2*	*his-1*	*aro-10*	*asp-1*	*trp-1*	*met-2*	
RV346(pSJ5)	24	71	Select			0		*str*
	0		16	Select				*str*
RV343(pSJ5)			Select			10		*str*
RV340(pSJ5)			11			Select	3	*str*
			<1		Select	18		*spc*
			Select		4	2		*str*

[a] About 200 recombinants were tested in each cross.

TABLE 8. *Gene transfer from El Tor donor to classical recipient RV169*

El Tor donor[a]	% Co-transfer to classical strain[b]		
	met-2	*trp-1*	*his-1*
RJ223(pSJ13)	32	65	Select
	41	Select	20
	Select	3	2

[a] Donors were counterselected with spectinomycin.
[b] About 200 recombinants of each class were tested.

imal to the presumed Tn*1* insertion at high frequencies. By this criterion, the location of Tn*1* insertions in all three donors was satisfactorily determined. Although the genes defined by the phenotypically similar mutations in the donors and recipients were almost certainly not allelic, the transfer properties of the donors, and the linkages obtained with them, indicated that they were located in the same chromosomal region. Because genes that control related biosynthetic functions are often clustered, this screening procedure appears generally useful for locating Tn*1* insertion sites.

Tfr donors of strain RV340 were unique in their ability to transfer genes in either direction from the Tn*1* insertion site. Our interpretation that this strain contained duplicate copies of the Tn*1* transposon in reverse orientation is supported by the work of Robinson et al. (21) who described plasmids that contained tandem insertions of Tn*1* in opposite orientations.

By selecting early markers on either side of the strain RV340 transfer origin, recombinants were obtained that arose mainly from one or the other of its donor classes. In these recombinants, the effects of polarized transfer were useful for helping determine gene orders. It was not possible, however, to determine the direction of transfer of late markers, and this ambiguity made strain RV340 less useful for analyzing distal markers.

Linkage analysis with Tfr classical donors yielded the circular map presented in Fig. 4. Except for the *rif* marker, the gene order was the same as that obtained in $P^+ \times P^-$ crosses. Perhaps the most significant difference between the two maps was the close linkage of the *his-1* and *pro-2* markers that was obtained with Tfr donors, but not with P^+ donors. The establishment of linkage between these two markers, together with the confirmation of previously determined linkages (20), provided evidence for the circularity of the *V. cholerae* genetic map. The reason for the lack of ability to detect linkage between the *pro*-2 and *his*-1 markers with P^+ donors is not known, but may indicate that a preferred site for P-factor chromosomal transfer exists in this region.

Johnson and Romig showed that most of the phenotypically similar mutations in El Tor strain RJ1 and classical strain 162 mapped in the same order (11). The results of the present study, which showed that El Tor Tfr donors transferred functionally equivalent genes at about the same frequency to either El Tor or classical recipients, provided support for the close genetic relatedness of these two biotypes. Mekalanos et al. (16) recently reported that mutations that affected toxin production in

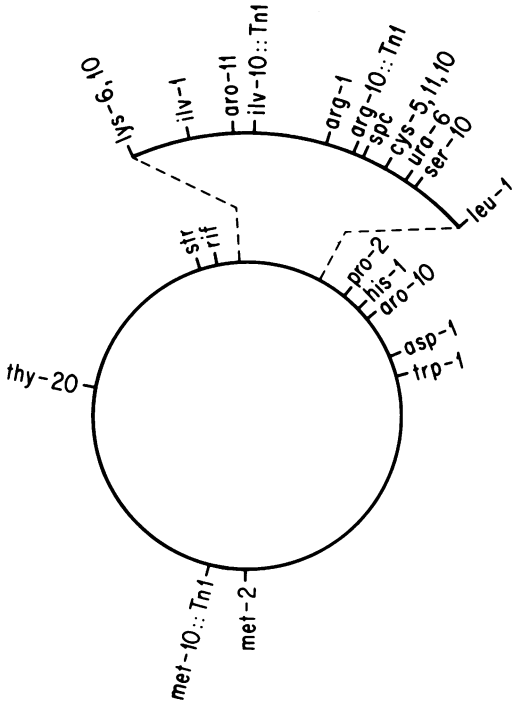

FIG. 4. *Circular genetic map of V. cholerae strain 162 with Tfr donors. Tn1 insertions are positioned relative to chromosomal markers. Relative distances are proportional to genetic linkages.*

classical stain 569B exerted similar effects after they were transferred to El Tor strain RJ1. It thus appears that gene exchange between these two *V. cholerae* biotypes occurs relatively freely and that direct genetic comparisons between them are feasible.

ACKNOWLEDGMENTS

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