Chromosome Mobilization in *Vibrio cholerae* (biotype eltor) Mediated by Sex Factor P

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SUMMARY

Crosses between P^+ strains of *Vibrio cholerae* (biotype 'eltor') and P^- strains of *V. cholerae* ('classical') were infertile under conditions in which $P^+ \times P^-$ crosses of *V. cholerae* ('classical') strains were fertile. The efficiency of conjugation, as determined by the frequency of P transfer, was lower with P^+ strains of the 'eltor' biotype compared with P^+ strains of *V. cholerae* ('classical'). However, incubation of P^+ strains at 44.5 °C, prior to mating, induced fertility in bacteria of the 'eltor' biotype and enhanced it in *V. cholerae* ('classical') crosses. It is suggested that new donors associated with a greater efficiency of conjugation are formed at 44.5 °C in P^+ strains of both biotypes.

INTRODUCTION

Genetic recombination in *Vibrio cholerae* is known to be mediated by a transmissible sex factor P (Bhaskaran, 1960, 1964; Bhaskaran & Sinha, 1971). Crosses between P^+ and P^- strains are fertile and analysis of recombinants showed unidirectional genetic transfer from P^+ to P^- strains (Bhaskaran, 1964). The P factor is also transmissible to strains of *V. eltor* as well as *V. cholerae* ('classical'). The former are distinguished from the latter by their haemolysis (hly⁺) of sheep red blood cells (Feeley & Pittman, 1963), resistance to a Group IV lytic phage (IV-r) (Mukerjee, 1963) and to polymyxin (pmx-r) (Gan & Tjia, 1963) and other traits. *V. cholerae* ('classical') and *V. eltor* are taxonomically indistinguishable because of a wide range of common characters and are now included in the species *V. cholerae* (Hugh, 1966). The term 'eltor' is retained here to designate *V. cholerae* strains which are either hly⁺ or IV-r because of the observed clear-cut genetic differences between *V. cholerae* ('classical') and strains of the 'eltor' biotype.

In this paper we describe the effects of incubation at 44.5 °C for 30 min on P^+ strains of both biotypes in crosses with P^- strains of *Vibrio cholerae* ('classical'). This temperature, although unfavourable for the growth of *Vibrio* strains, does not greatly affect viability. Earlier studies at 42 °C, which permitted growth of *V. cholerae*, and at 50 °C, which considerably reduced viability, were ineffective.

METHODS

Bacterial strains. Of the strains used (Table 1), KB9, KB16 and KB460 were derivatives of *Vibrio cholerae* ('classical') strain 162 (Bhaskaran, 1960), whereas KB408 and KB440 were P^+ derivatives of 'eltor' strains kindly supplied by Dr D. Barua (World Health Organization, Geneva) and Dr A. K. Thomas (Central Research Institute, Kasauli, India) respectively.

Culture media. DNB (Difco Nutrient Broth: Difco Laboratories, Detroit, Michigan,

Table 1. *Vibrio cholerae* strains

Strain no.	Previous designation	Sex factor	<i>ilv</i>	<i>arg</i>	<i>his</i>	<i>pur</i>	<i>leu</i>	<i>str</i>	<i>hly</i>	<i>pmx</i>	ϕ -IV	O anti-genic type	Biotype
KB9	V 58:SR	P ⁻	—	—	—	+	+	R	—	S	S	O-Og	'classical'
KB16	V 63 P ⁺	P ⁺	+	+	+	—	—	S	—	S	S	O-In	'classical'
KB460	—	P ⁻	—	—	—	+	+	R	—	S	S	O-In	'classical'
KB408	T 50 P ⁺	P ⁺	+	+	+	+	+	S	—	R	R	O-Og	'eltor'
KB440	—	P ⁺	+	+	+	+	+	S	+	R	R	O-Og	'eltor'

Abbreviations: *ilv*, isoleucine + valine; *arg*, arginine; *his*, histidine; *pur*, purines; *leu*, leucine; *str*, streptomycin; *hly*, haemolytic activity; *pmx*, polymyxin; ϕ -IV, Group IV lytic phage; O-Og, O antigenic type Ogawa; O-In, O antigenic type Inaba.

— = absence sex factor or haemolytic activity or nutritional requirement. R and S = resistance or sensitivity.

U.S.A.) was prepared by dissolving 16 g in 1000 ml of a 0.5 % solution of NaCl and BHI (Brain Heart Infusion: Difco Laboratories) by dissolving 37 g in 1000 ml of distilled water. Semisolid (sloppy) and solid media contained respectively 0.4 and 1.0 % agar (Oxoid, no. 3). The minimal medium was that of Bhaskaran & Sinha (1971). All media were sterilized at 15 lb/in² for 10 min.

Crosses. Crosses were performed as described earlier (Bhaskaran & Sinha, 1971) except that, when required, subcultures were incubated for a further 30 min at 44.5 °C.

To determine the persistence of the 44.5 °C temperature effect on P⁺ strains, 0.5 ml amounts were transferred to 9.5 ml fresh BHI and incubated at 37 °C. Viable counts were made at 30 min intervals and samples taken for crosses with appropriate P⁻ strains. In these crosses, as in controls, 10⁸ to 10⁹ donor (P⁺) organisms were mixed with about 10¹⁰ organisms of the recipient (P⁻) and the suspensions passed through membrane filters ('Metricel', Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.; pore size, 0.2 µm). These filters were placed on the surface of BHI agar plates and incubated at 37 °C for 60 min. Mated organisms were then suspended in minimal medium and streaked on selective minimal agar for the isolation of recombinants. In this study, the donor selective marker was either *ilv*⁺ or *arg*⁺ with *str*-r as the recipient selective marker.

Test for P factor. For gross differentiation of P⁺ and P⁻ strains, cross-matching tests were performed in semisolid agar (Bhaskaran & Sinha, 1971). P⁺ strains showed a growth-thinning effect on indicator P⁻ strains but not on P⁺ strains. P⁻ strains had no effect on either strain.

For the assay of P⁺ bacteria (Bhaskaran, 1964), 0.5 ml of tenfold dilutions of the test culture were mixed with 0.2 ml of a 3 h BHI culture of KB9 (P⁻) in 7 ml of soft agar (DNB) on the surface of DNB agar plates. When the soft agar had set, the plates were inverted and incubated overnight at 37 °C. The number of P⁺ organisms was correlated with the number of clearings (infectious centres) observed.

This procedure was modified when the efficiency of conjugation (as indicated by transfer of P factor) between P⁺ and P⁻ strains of *Vibrio cholerae* was determined. Streptomycin-sensitive P⁺ and streptomycin-resistant P⁻ organisms were mated on membranes and the numbers of newly infected P⁺ organisms assayed after 30 min contact. For this purpose the organisms were suspended in DNB and assayed for P⁺ cells as described above using the parental P⁻ strain as indicator. In these tests, the soft overlay as well as the basal DNB agar contained streptomycin sulphate (500 µg/ml) to inhibit further spread of P factor from parental P⁺ to indicator P⁻ strain during the overnight incubation required for the development of discrete clearings (infectious centres). The same P⁺ and P⁻ strains were used in control experiments but without mating on membranes.

Table 2. Genetic transfer from P^+ strains of *Vibrio cholerae* (biotype 'eltor') to P^- strain KB460 of *V. cholerae* ('classical'); effect of incubation of the donor strain at 44.5 °C for 30 min

Strain		3 h BHI culture at 37 °C (control)	Time of incubation (min) at 37 °C after keeping for 30 min at 44.5 °C					
			0	30	60	90	120	150
KB440 P^+ ('eltor')	Viable count/ml	9.00×10^8	3.60×10^7	4.00×10^7	8.90×10^7	3.60×10^8	6.40×10^8	1.08×10^9
	No. of generations	—	—	0.15	1.31	3.32	4.15	4.91
	No. of recombinants/ 10^8 donor bacteria							
	<i>arg</i> ⁺ <i>str-r</i>	Nil	108	913	391	43	9	4
	<i>ilv</i> ⁺ <i>str-r</i>	Nil	135	1158	395	34	13	7
KB408 P^+ ('eltor')	Viable count/ml	8.38×10^8	2.20×10^7	3.10×10^7	5.20×10^7	7.20×10^7	1.84×10^8	5.30×10^8
	No. of generations	—	—	0.50	1.24	1.71	3.06	4.61
	No. of recombinants/ 10^8 donor bacteria							
	<i>arg</i> ⁺ <i>str-r</i>	7	321	1021	1852	861	122	26
	<i>ilv</i> ⁺ <i>str-r</i>	2	139	1037	1590	814	165	58

RESULTS

Crosses between P^+ strains of *Vibrio eltor* and P^- strains of 'classical' biotype. No recombinants were obtained when KB440 ('eltor', P^+) was mated with KB460 ('classical', P^-) when both strains were grown at 37 °C prior to mating (Table 2). Following incubation of the donor strain at 44.5 °C for 30 min, there was an immediate appearance of fertility which reached its peak 30 min after subsequent incubation at 37 °C and then declined rapidly until after 150 min the cross was practically infertile. As the mating took place on a membrane during incubation at 37 °C for 60 min, the period of maximal fertility is likely to be between 30 and 90 min after incubation at 44.5 °C. This would correspond to about three generations on the assumption that the growth of P^+ bacteria on the membrane was similar to that in BHI. However, this might not be the case because F^+ strains of *Escherichia coli* stopped multiplying when mixed in 1:10 ratio with F^- bacteria (Evenchik, Stacey & Hayes, 1969). Recombinant frequencies with samples taken after 60 min (when the population had just doubled) showed a sharp decline; thus it is clear that the donor state was fully expressed before the first cell division but not later. The donor state was therefore not heritable. This finding is in fact very similar to ultraviolet induction of chromosome transfer in *E. coli* (Evenchik *et al.* 1969) where the induced donor state can be attributed to the formation of a genetic structure which can lead to transfer but cannot replicate.

Crosses between another 'eltor' P^+ strain, KB 408 and the same P^- strain ('classical'), KB460, gave similar results (Table 2). In this case, maximal fertility was seen at 60 min after incubation at 44.5 °C (after one generation) but decline commenced before the end of the second generation. A low degree of residual fertility was retained after 150 min (4.61 generations). The few colonies which appeared in controls were probably revertants of the P^- strain.

These observations were confirmed in three repetitions of each of the above two crosses and on no occasion did maximum fertility extend beyond 60 min after incubation at 44.5 °C. To facilitate interpretation of these findings, the effect of incubating the donor at 44.5 °C in crosses between P^+ and P^- strains of 'classical' *Vibrio cholerae*, of known fertility, was

Table 3. Genetic transfer from P^+ strain KB16 of *Vibrio cholerae* ('classical'), to P^- strain KB460 of *V. cholerae* ('classical'); effect of incubation of the donor strain at 44.5 °C for 30 min

	3 h BHI culture at 37 °C (control)	Time of incubation (min.) at 37 °C after 30 min at 44.5 °C					
		0	30	60	90	120	150
Viable count/ml	5.90×10^8	2.80×10^7	3.20×10^7	5.80×10^7	1.55×10^8	3.20×10^8	4.40×10^8
No. of generations	—	—	0.19	1.05	2.47	3.52	3.97
No. of recombinants/ 10^8 donor bacteria							
<i>arg^+ str-r</i>	176	778	2796	3469	945	541	418
<i>ilv^+ str-r</i>	197	1071	2572	2545	804	419	527

Table 4. Efficiency of P transfer to P^- strain KB9 of *Vibrio cholera* ('classical') in crosses; effect of incubation of donor strains at 44.5 °C for 30 min

Donor (P^+)	Donor incubation temperature	Efficiency of P transfer*	
		Expt 1	Expt 2
KB16 ('classical')	4 °C (control)	200.6	119.6
	44.5 °C	428.1	164.4
KB408 ('eltor')	4 °C (control)	17.2	12.3
	44.5 °C	34.8	17.5
KB440 ('eltor')	4 °C (control)	30.5	23.0
	44.5 °C	83.1	92.8

* The efficiency of P transfer was calculated as the no. of clearings (infectious centres)/100 viable donor organisms.

studied. Recombination frequencies were higher immediately after incubation of the P^+ strain at 44.5 °C with a maximum at the time of the first cell division (Table 3). This means that even in cultures containing competent donors, their relative numbers were increased after incubation at 44.5 °C. This is in contrast to 'eltor' P^+ strains in which such competent donors only appeared after incubation at 44.5 °C.

A possible explanation for the induced donor activity in 'eltor' P^+ strains incubated at 44.5 °C. There could be two explanations for the failure of 'eltor' P^+ strains grown at 37 °C to yield recombinants: conjugation between 'eltor' P^+ and 'classical' P^- strains did not occur to any significant extent and/or chromosomal transfer did not take place. As the P factor is transmissible by conjugation, the frequency of its transfer should indicate the efficiency of conjugation. This was determined in crosses between three streptomycin-sensitive P^+ strains and a streptomycin-resistant P^- strain on membrane filters. To permit every P^+ cell to conjugate with at least one P^- cell, P^+ and P^- bacteria were mated in the ratio of 1:10.

Conjugation between *Vibrio cholerae* ('classical') strains was highly efficient and P transfer was in excess of 100 % after 30 min contact on the membrane (Table 4). Incubation at 44.5 °C for 30 min (and subsequent incubation at 37 °C for 60 min) gave even higher values. With 'eltor' P^+ strains conjugation was less efficient; incubation of KB408 at 44.5 °C effected a slight increase whereas with strain KB440 the effect was greater, approaching 100 %. In control experiments with the same suspensions of streptomycin-sensitive P^+ and

Table 5. Frequency of transfer of P factor to P⁻ strain KB9 of *Vibrio cholerae* ('classical') in soft agar containing streptomycin lethal to the donor (P⁺) strains

P ⁺ strain	Expt no.	Viable count of P ⁺ bacteria/ml	No. of infectious centres/ml	% frequency of P transfer
KB 408 ('eltor')*	1	1.24×10^8	3.60×10^5	0.29
	2	1.07×10^8	4.90×10^5	0.46
KB 440 ('eltor')*	1	1.59×10^8	5.30×10^5	0.33
	2	1.63×10^8	1.50×10^6	0.92
KB 16 ('classical')	1	1.32×10^8	7.20×10^5	0.55
KB 16 ('classical')*	1	1.17×10^8	1.43×10^6	1.22

* Cultures incubated at 44.5 °C for 30 min.

streptomycin-resistant P⁻ strains without mating on a membrane, P transfer was low (0.29 to 1.22 %; Table 5) in the presence of streptomycin. This confirmed that the frequencies shown in Table 4 reflect the efficiency of P transfer by conjugation.

The P transfer in excess of 100 % observed with 'classical' *Vibrio cholerae* KB16 could have resulted from either a further spread of P factor from newly infected P⁺ bacteria or multiple rounds of conjugation between a P⁺ bacterium and several P⁻ bacteria. On the other hand, multiplication of parental and newly infected P⁺ organisms could have been involved. Furthermore, 'eltor' P⁺ strains were not totally deficient in conjugational ability. Although frequency of conjugation was less than with 'classical' P⁺ strains (12.3 to 30.5 %), conjugation did occur in the two strains studied even without incubation at 44.5 °C.

DISCUSSION

Genetic recombination between two strains of *Vibrio cholerae* ('eltor' biotype) and a strain of *V. cholerae* ('classical') showed certain interesting features which distinguished them from crosses between two *V. cholerae* ('classical') strains. The P⁺ 'eltor' strains were inefficient as gene donors to a P⁻ 'classical' *V. cholerae* strain under normal conditions of mating but incubation of the former at 44.5 °C induced transient increased levels of fertility (Table 2). 'Classical' P⁺ strains were generally fertile in crosses with P⁻ strains of *V. cholerae* ('classical') but recombination frequencies increased after incubation of the donor strain at 44.5 °C (Table 3). No appreciable differences were observed by incubating recipient P⁻ strains at 44.5 °C. Reverse crosses, i.e. mating P⁻ 'eltor' with P⁺ 'classical' *V. cholerae*, seem to be fertile without incubation of either strain at 44.5 °C. All P⁻ × P⁻ crosses were infertile and unaffected by incubation at 44.5 °C.

Efficiency of conjugation as indicated by frequency of P transfer was greater when both P⁺ and P⁻ strains were of the 'classical' biotype than between 'eltor' P⁺ and 'classical' P⁻ strains (Table 4). Had this been the only reason for the difference in fertility between the two crosses, one would expect reduction in recombination frequency but not its absence. Incubation at 44.5 °C did enhance efficiency of conjugation three to fourfold but this cannot account for the considerable increase of gene donor activity in 'eltor' strains. Some form of chromosomal mobilization appears to occur in these strains at 44.5 °C. It may be that DNA strand breaks occur at 44.5 °C (Bridges, Ashwood-Smith & Munson, 1969) resulting in a transient association between the sex factor P and bacterial chromosomal segments in 'eltor' strains. Such events probably occurred normally in 'classical' *Vibrio cholerae* strains even at 37 °C. As proposed for *Escherichia coli* (Evenchik *et al.* 1969) such transient

structures may be transferred by conjugation from bacterium to bacterium leading to genetic recombination.

Analysis of unselected markers in the recombinants isolated has been unsatisfactory because of the low incidence of linkage in *Vibrio cholerae* (Bhaskaran, 1964). Thus linkage differences between the induced donor state at 44.5 °C and in normal donors at 37 °C in P⁺ strains of 'classical' *V. cholerae* cannot be detected.

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