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Vibrio cholerae Hybrid Sex Factor That Contains Ampicillin Transposon Tn*1*

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The ampicillin resistance transposon Tn*1* was translocated from the R plasmid RP4 to the *Vibrio cholerae* conjugative plasmid, P. The hybrid sex factor P::Tn*1* was highly transmissible and expressed the biological activities of the P factor. In addition, P::Tn*1* facilitated transfer of RP4 to *V. cholerae* recipients. Physical studies of P::Tn*1* indicated that the Tn*1* transposon was added to the otherwise unaltered P plasmid.

The *Vibrio cholerae* conjugative plasmid, P, is similar in several respects to the F plasmid of *Escherichia coli*. Both can exist as autonomous replicons capable of derepressed self-transfer, and they also mediate low-frequency transfer of bacterial genes (2, 7, 22). However, unlike F, integration of the P plasmid into the bacterial chromosome has not been observed (4, 21), and treatments that readily eliminate F do not have detectable effects on P (21). The P factor does not alter the phenotype of bacteria which contain it, except that P^+ *V. cholerae* can be identified by the clear areas, or "lacunae," which they produce on a lawn of recipient P^- bacteria (2,22).

Datta et al. (4) reported that the P factor could be extracted as a covalently closed circular DNA molecule with a molecular weight of 80×10^6 and a buoyant density in CsCl of 1.702 g/cm^3. These investigators also demonstrated that *V. cholerae* mating strains contain a 40-megadalton (Mdal) cryptic plasmid whose buoyant density, 1.707 g/cm^3, is the same as that of the *V. cholerae* chromosome.

During studies on fertility, we transferred several plasmids to *V. cholerae*, where their properties and possible interactions with the P factor were determined. The ampicillin (Ap) resistance transposon, Tn*1* (9, 10, 12), was readily transposed from the broad host range R plasmid RP4 (6, 8, 18, 20) to the *V. cholerae* sex factor. The resulting hybrid sex factor, P::Tn*1*, retained the biological properties of the P factor. The physical properties of P::Tn*1* were also compared with those of the P factor and the cryptic plasmids. The results indicated that Tn*1* was inserted into the intact P factor and showed that the *V. cholerae* plasmids are smaller than previously reported (4).

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MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are described in Table 1.

Media. Nutrient and synthetic media, diluents, and procedures for plating bacteria with soft agar have been described elsewhere (21, 22). Antibiotics for selection and counterselection were used at the following concentrations: 100 μg of streptomycin, 100 μg of spectinomycin, 50 μg of nalidixic acid, 300 μg of penicillin G or Ap, 25 μg of kanamycin (Km), and 10 μg of tetracycline (Tc) per ml.

Mating procedures. The mating procedures have been described elsewhere (21, 22). Briefly, donor and recipient cultures were grown in brain heart infusion broth (Difco) until a cell density of about 10^8 to 5×10^8 cells per ml was obtained. Recipients were vigorously agitated during cultivation, whereas donors were gently shaken. The bacteria were mixed at a donor: recipient ratio of 1:10 and incubated with gentle agitation for 150 min. After mating, samples were plated directly onto selective media to determine resistance transfer. When nutritional markers were selected, the mixture was centrifuged and resuspended in the same volume of half-strength brain heart infusion broth before samples were spread onto minimal agar.

Qualitative matings. The donor abilities of large numbers of isolates or colonies were qualitatively determined by the replica mating procedure of Low (14).

Plasmid stability. The stability of plasmid-borne antibiotic resistance genes or the continued capacity to transfer resistance was tested by sequentially subculturing small inocula of the various strains in nonselective brain heart infusion broth. At frequent intervals, isolated colonies were transferred into a grid on master plates and replicated onto antibiotic media. Retention of transfer ability was determined by replicating the bacterial growth onto lawns of recipient bacteria (14). P^+ bacteria were detected by lacunae assays (22).

Growth of strains for labeling DNA. The strains

TABLE 1. *Bacterial strains and plasmids*

Strain/plasmid	Relevant properties[a]	Reference/source
E. coli		
J53	*metF proA*	From N. Datta (5).
V. cholerae		
RV106	*arg-1 his-1 ilv-1 nal*	The P^- strains are all derivatives of Bhaskaran's strain 162 (2, 21).
RV31	*arg-1 his-1 ilv-1 str*	
RV34	*leu-1 pur-1*	
RV127	*leu-1 pur-1 pro-1 mal-1 suc-1 str*	
RV160	*arg-1 his-1 ilv-1 spc*	
RV169	*arg-1 his-1 ilv-1 met-2 trp-1 str*	
RV107	*arg-1 his-1 ilv-1 nal* (P^+)	P factor transferred to RV106 from Bhaskaran's strain V58(P^+) (21).
Plasmids		
P	Conjugative plasmid, lacunae forming	
RP4	Ap^r Km^r Tc^r	
P::Tn*1*	Ap^r, lacunae forming	This paper

[a] Symbols for bacteria and plasmids are according to Bachmann et al. (1) and Novick et al. (17), except *str, spc,* and *suc-1* (21).

were diluted and subcultured several times in medium A broth (16) supplemented with 1.0% Casamino Acids (Difco), 50 μg of tryptophan, 250 μg of deoxyadenosine, and other required amino acids, purines, and pyrimidines at 50 μg/ml. A 30-ml logarithmically growing culture containing 3 μCi of [*methyl*-^{3}H]thymidine (Schwartz/Mann, 6,000 mCi/mmol) per ml was incubated for about 150 min at 37°C on a rotary shaker.

Isolation of plasmid DNA. Labeled cultures were harvested, lysed, and enriched for plasmid DNA from "cleared lysates," and for most purposes covalently closed plasmid DNA was purified by dye-buoyant density equilibrium centrifugation, fractionated, and counted by the procedures described by Clewell and Helinski (3). Ethidium bromide was extracted from the pooled plasmid fractions with isopropanol, and CsCl was removed by extensive dialysis against TES buffer (3). Larger amounts of plasmid DNA were prepared from the cleared lysate supernatant fluid from 400-ml cultures by the procedure of Humphreys et al. (11) and further purified by dye-buoyant density centrifugation.

Sucrose density gradient centrifugation. The methods used for determining the sedimentation rates of supercoiled DNA fractions were described by Clewell and Helinski (3). Linear sucrose gradients (5 to 20%) were centrifuged for 55 min at 40,000 rpm in an SW40 rotor, and 6-drop fractions were collected and counted. For some experiments, plasmid DNA from cleared lysates was further purified by similar procedures, except the fractions were collected into tubes, and one of the 6 gradients was fractionated to locate the plasmid peak.

Electron microscopy. Samples of purified plasmid DNA were spread by the "microversion" modification of the spontaneous adsorption method of Lang (13), except that the spreading mixture contained 40% formamide and the grids were stained with uranyl acetate (10) before they were shadowed. Specimens were examined and photographed with an Hitachi Hu 11A electron microscope. Magnification was determined by calibration with a diffraction grating (Pelco), and bacteriophage PM2 DNA was included in all samples as an internal control for length determinations.

Heteroduplex analysis. Plasmid DNA purified by dye-buoyant density centrifugation was exposed to 1,560 rads of gamma radiation to generate single-stranded breaks (23). After irradiation, the dye and CsCl were removed by isopropanol extraction and dialysis as previously described. Heteroduplex molecules were obtained by the methods of Heffron et al. (10), except that the DNA was reannealed by allowing it to stand for 7 h at 25°C after it was neutralized. Bacteriophage PM2 DNA and phage M13 single-stranded DNA were added to the reannealed DNA, and samples were mounted and examined as previously described. DNA contour lengths were obtained by tracing micrographs at 10 × magnification on an optical comparator, and the lengths of the tracings were determined with a map measuring device.

CsCl density gradient analysis. Buoyant density was measured in the analytical ultracentrifuge by the methods of Mandel et al. (15) with *Micrococcus lysodeikticus* DNA at a density of 1.731 g/cm^3 as reference.

RESULTS

E. coli J53(RP4) was mated with *V. cholerae* strains RV107(P^+) and RV106(P^-) by selecting transconjugants with Km and counterselecting against the *E. coli* donor with nalidixic acid. Resistant colonies were replicated to Ap and Tc nutrient agar plates to verify RP4 transfer. *E. coli* J53(RP4) transferred RP4 equally well to P^+ or P^- recipients. In addition, P^+ cells that acquired RP4 retained the ability to produce lacunae, and RP4 did not confer this ability to P^- bacteria (Table 2, cross 1 and 2).

Transfer of RP4 by P^+ and P^- *V. cholerae*. The P^- *V. cholerae* transferred RP4 to RV160(P^-) recipients at about the same frequency that the R plasmid was transferred to them by *E. coli* J53(RP4). However, P^+ bacteria transferred RP4 resistance genes considerably more efficiently than P^- bacteria, and the various resistance genes were transferred at widely different frequencies (Table 2, crosses 3 and 4). One class of transconjugants, selected with Ap, appeared at high frequency (8×10^{-1}), but none contained the other RP4 resistance markers. Another class of transconjugants, selected with Km (or Tc), occurred less frequently (10^{-4}), but contained all three RP4 resistance markers. Both kinds of RV160 transconjugants produced typical P^+ lacunae. The Ap^r Km^s Tc^s RV160 transconjugants transferred Ap^r and lacunae-forming ability to RV31 recipients at frequencies indistinguishable from the self-transfer frequency of the P plasmid (22); the transfer pat-

TABLE 2. *Transfer of RP4 resistance by E. coli and V. cholerae donors*

Donor	*V. cholerae* recipient	RP4 marker selected[a]	Transfer frequency[b]	Phenotype of transconjugants[c]		
				Nonselected RP4 marker	P factor	Inferred contents
E. coli						
J53(RP4)	RV106(P^-)	Km^r	1×10^{-6}	All	−	RP4
J53(RP4)	RV107(P^+)	Km^r	2×10^{-6}	All	+	P, RP4
V. cholerae						
RV106(P^-)(RP4)	RV160(P^-)	Ap^r	9×10^{-6}	All	−	RP4
		Km^r	3×10^{-6}	All	−	RP4
RV107(P^+)(RP4)	RV160(P^-)	Ap^r	8×10^{-1}	None	+	P::Tn*1*
		Km^r	1×10^{-4}	All	+	P, RP4
RV160(P::Tn*1*)	RV31(P^-)	Ap^r	>1	None	+	P::Tn*1*
RV160(P^+)(RP4)	RV31(P^-)	Ap^r	1	None	+	P::Tn*1*
		Km^r	2×10^{-3}	All	+	P, RP4

[a] Donors were counterselected with alternative antibiotics (Table 1).

[b] Transconjugants/input donor.

[c] From 45–100 transconjugants were scored for acquisition of both unselected RP4 markers; the P factor was detected by lacunae assays (22).

tern of the Ap^r Km^r Tc^r transconjugants was similar to that of the original RV107(P^+)(RP4) donors (Table 2, crosses 5 and 6). These results indicated that RP4 transfer was facilitated by P^+ bacteria. They also suggested that the Tn*1* transposon had been translocated from the poorly transmissible RP4 plasmid to form a freely transmissible P::Tn*1* hybrid. The physical studies presented in later sections support this interpretation.

Properties of *V. cholerae* (P::Tn*1*) strains. The stability of the presumed P::Tn*1* plasmid was determined by sequentially subculturing five independently derived RV160(P::Tn*1*) isolates in brain heart infusion broth without selection. No Ap^s segregants were detected (156 colonies tested) after more than 100 cell doublings. Most RV160(P^+)(RP4) transconjugants were also stable, and after 100 cell doublings, only 1 to 3% of the cells lost either RP4 or the P factor. A few isolates were less stable, and some segregated up to 13% antibiotic-sensitive cells within 10 generations of growth.

Sex factor activity of the P::Tn*1* plasmid was determined. Recombination frequencies for the selected auxotrophic markers (about 10^{-5} per donor) were not significantly different from those obtained in $P^+ \times P^-$ crosses (21).

Strain RV160(P::Tn*1*) was crossed to P^+ and P^- recipients, and transconjugants were selected with Ap. P::Tn*1* was transferred 60% less frequently to P^+ than to P^- recipients, presumably as a result of the incompatibility of the two plasmids.

The P factor and P::Tn*1* were transferred into RP4-containing strains which were immediately mated to RV31(P^-) recipients. Transconjugants from these matings were selected for RP4 markers. The results (Table 3) indicate that RP4 transfer is probably not facilitated by the P factor, and that P::Tn*1* is primarily responsible for the enhanced transfer.

Molecular weights of plasmids. The P factor, P::Tn*1* and RP4 were separately introduced into *V. cholerae* RV106(P^-), and the resulting cultures were labeled with [^{3}H]thymidine. The uninfected RV106 culture was also labeled and examined by the same procedures. Radioactive plasmid DNA from each strain was purified by dye-buoyant density centrifugation and examined by velocity sedimentation through 5 to 20% neutral sucrose gradients. Bacteriophage T7 [^{14}C]DNA was included in all determinations and assigned a sedimentation value of 32S and a molecular mass of 25 Mdal (24). Most of the ^{3}H-labeled plasmid DNA sedimented at a position corresponding to the covalently closed circular DNA molecules of the transmissible plasmids. In addition to the major, fast sedimenting peak, several, more slowly sedimenting peaks were also detected. These were determined by velocity sedimentation through alkaline sucrose gradients to correspond to plasmid molecules that contained single-stranded interruptions ("nicked," circular DNA) and to the cryptic plasmid in these strains (4).

Characteristics of the cryptic plasmid determined from DNA purified from the RV106(P^-) control culture corresponded well to those obtained with samples that contained the transmissible plasmids. The sedimentation rate and corresponding molecular mass of the *V. cholerae* plasmids are presented in Table 4. The value of 64S that we obtained for RP4 in *V. cholerae* agrees with previously reported values of 63 to 64S (6, 8, 18). However, our 69S and 54S sedi-

TABLE 3. *Comparison of the ability of P or P::Tn1 to mobilize RP4*

Superinfecting plasmid[a]	Percent of RV106(RP4) receiving P or P::Tn*1*[b]	No. of Km^r RV31 transconjugants per ml
P::Tn*1*	9	5.2×10^3
P	6.5	2.3×10^1

[a] RV106(RP4) was superinfected with either P or P::Tn*1* by mating to the appropriate donor for 90 min. The mating mixture was diluted and incubated to yield logarithmically growing cells. Each culture was then mated to RV31 to determine the ability of superinfected RV31 to transfer the RP4 Km^r marker.

[b] The fraction of each RV106(RP4) culture converted to the P$^+$ state was determined by lacunae assays (21) at the time it was mated to RV31. Average of 2 experiments.

mentation values for the P factor and cryptic plasmid differ significantly from the values of 84S and 58S that have been previously reported (4).

Contour lengths of plasmids by electron microscopy. Independent estimates of the sizes of these plasmids were determined by electron microscopy. The contour lengths of the plasmids (Table 4) agreed with values calculated from the centrifugation experiments. These measurements indicated that P::Tn*1* was 4.3 Mdal larger than the P factor, and thus agreed well with the size difference calculated from the sedimentation rates. The contour measurements also confirmed our molecular mass determinations for the P factor (47 Mdal) and for the cryptic plasmid (25 Mdal). Molecules larger than the 52-Mdal P::Tn*1* plasmid were not observed in these preparations. The cryptic plasmid was observed in both the P$^+$ and P$^-$ strains, but was not detected in RV106(P::Tn*1*). Of additional interest, small numbers of a 3.2-Mdal plasmid were observed in all of these electron microscopic preparations, but were not detected in the centrifugation experiments.

Properties of plasmids purified by sucrose gradient velocity sedimentation. Properties of plasmid DNA obtained from cleared lysates and further purified by velocity sedimentation through sucrose were also examined. The DNA in these samples sedimented at 83S instead of the 69S obtained for P factor DNA purified in ethidium bromide-cesium chloride. However, the electron microscopic contour lengths of the P factor molecules in both preparations were identical (Table 4). If these preparations or the unfractionated cleared lysates were exposed to 0.5% sodium dodecyl sulfate, the 83S DNA was not detected. Instead, about 10% of the labeled DNA sedimented at 69S, and the remainder sedimented at 54S, the expected position for nicked, circular 47-Mdal DNA.

TABLE 4. *Properties of V. cholerae plasmids*

Plasmid	Sedimentation rate (S)	Length (μm)	No. of molecules	Molecular mass[a] (Mdal)	Density (g/cm^3)
Cryptic I		1.5 ± 0.3	18	3.2	1.707
Cryptic II	54	11.9 ± 0.6	12	24.7	1.707
P	69	22.9 ± 0.9	10	47.4	1.703
P::Tn*1*	73	25.0 ± 0.9	12	51.7	1.703
P	83[b]	23.1 ± 0.6[b]	14	47.6	

[a] Molecular mass was computed from contour lengths relative to an internal standard of bacteriophage PM2 DNA of molecular mass 6.5 Mdal (23).

[b] The sedimentation value and contour lengths were determined on preparations purified from cleared lysates on neutral sucrose gradients (see text).

Plasmid buoyant densities. The buoyant densities of the P factor and the cryptic plasmids were essentially identical to previously reported values (4), and the density of P::Tn*1* was not detectably different from the P factor (Table 4).

Heteroduplex analyses. As illustrated in Fig. 1, the single-stranded loop corresponding to the Tn*1* transposon was detected on the heteroduplexes formed between P and P::Tn*1*. The insertion loop measured on nine heteroduplex molecules was calculated to contain about 1.7 Mdal of DNA; the stalk region formed by the inverted repeats at each terminus of the Tn*1* transposon contained about 150 base pairs (9). Single-stranded deletion loops were not observed on the heteroduplex molecules.

DISCUSSION

The Ap transposon, Tn*1*, was frequently translocated from the resistance plasmid, RP4, to the *V. cholerae* sex factor, P. Bacteria that contained P::Tn*1* exhibited the biological activities of P$^+$ donors, including (i) derepressed, high frequency plasmid transfer; (ii) ability to form lacunae; (iii) chromosomal gene transfer; and (iv) stable plasmid maintenance. The Ap^r bacteria derived from P::Tn*1* transconjugants transferred Ap^r and produced lacunae, indicating that Ap^r reliably detects the hybrid P factor in these strains. The physical evidence that Tn*1* was added to, rather than substituted for, P factor sequences was consistent with these biological properties.

The reconstruction experiments suggested that P::Tn*1*, rather than P, is primarily responsible for the facilitated transfer of RP4, but the mechanism for this was not determined. The possibility was tested that P::Tn*1* and RP4 recombined through their homologous Tn*1* sequences to form a transmissible, cointegrate

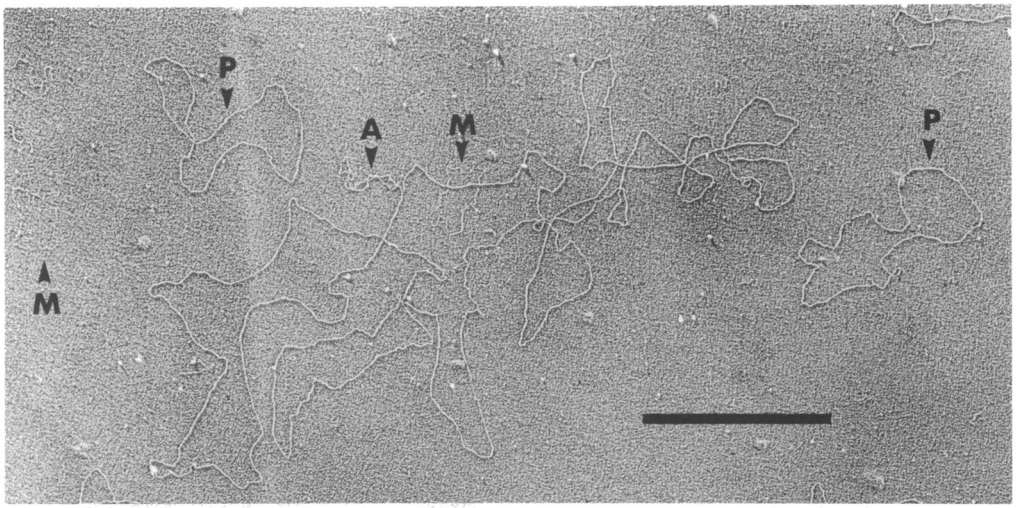

FIG. 1. *Heteroduplex between the hybrid plasmid, P::Tn1, and the P factor. The arrow at A indicates the single-stranded insertion loop of the ampicillin transposon, Tn1; P indicates the double-stranded PM2 phage DNA; and M indicates single-stranded M13 phage DNA. The bar equals 1.0 μm.*

plasmid (19); but no hybrid plasmids other than P::Tn*1* were identified.

The sedimentation studies suggest that under some conditions, P factor DNA is released as a "relaxation complex" (3) and that further purification (e.g., dye-buoyant density centrifugation) yields mostly uncomplexed, covalently closed molecules. The cleared lysis procedure (3) may release P factor DNA in a constrained, rapidly sedimenting configuration similar to the "nucleoid bodies" reported for *E. coli* (25). If so, the lower 69S sedimentation rate obtained with ethidium bromide-cesium chloride purification might result from relief of the constraint to give an unfolded, but still supercoiled form of P.

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