ACTIVITY OF PLASMID REPLICONS IN *CAULOBACTER CRESCENTUS*: RP4 AND ColE1

EDWARD A. O'NEILL, CRAIG BERLINBERG and ROBERT A. BENDER[1]

Department of Cellular and Molecular Biology, Division of Biological Sciences, University of Michigan, Ann Arbor, Michigan 48109

Manuscript received August 2, 1982
Revised copy accepted December 8, 1982

ABSTRACT

The RP4 replicon was detected as covalently-closed circular DNA in *Caulobacter crescentus* strains into which it had been transferred from *Escherichia coli*. RP4-mediated transfer of ColE1-associated markers into *C. crescentus* occurred, but only as the result of transposon-mediated events. Both transposition of a ColE1-associated marker onto RP4 and cointegration of ColE1 with RP4 were observed. Chimeric plasmids containing both a ColE1 and an RP4 origin of replication were stably maintained in *C. crescentus*, but similar plasmids lacking the RP4 origin of replication were not stably maintained in *C. crescentus*. Thus we show that the ColE1 replicon cannot be maintained in *C. crescentus* unless it is covalently linked to another replicon, such as RK2, that can be maintained.

CAULOBACTER CRESCENTUS is a dimorphic bacterium with a well defined life cycle and has been proposed as a model system for studying differentiation (e.g., BENDER, AGABIAN and SHAPIRO 1980). Cell division in *C. crescentus* is asymmetric, giving rise to two daughter cells which differ physiologically as well as morphologically (POINDEXTER 1964). The stalked daughter replicates its chromosome immediately after cell division whereas the flagellated ("swarmer") daughter must first mature, a process requiring about a third of a generation time (DEGNEN and NEWTON 1972). This delay in DNA replication during swarmer cell differentiation and the subsequent initiation of replication that occurs when the swarmer cell has matured into a stalked cell have been generally taken as important regulatory events in the *C. crescentus* cell cycle (OSLEY and NEWTON 1978; SHEFFEREY and NEWTON 1981; SHAPIRO 1976).

We began an analysis of plasmid replication in *C. crescentus* in an attempt to find a replicon smaller than the chromosome that would serve as a model system to study this unique pattern of DNA replication. Genetic analysis of the transfer of antibiotic resistance had been interpreted previously as demonstrating transfer and independent maintenance of the plasmids RP4 and ColE1 in *C. crescentus* (ALEXANDER and JOLLICK 1977; ELY 1979). However, there has been no physical evidence for the presence of these plasmids as independent replicons in *C. crescentus*. The work presented here confirms the transfer of RP4 to *C. crescentus* and demonstrates its independent existence as a low copy number,

[1] To who correspondence should be directed.

covalently closed, circular plasmid. On the other hand, we show that ColE1 does not function as a replicon in *C. crescentus* by itself but can be maintained if it is covalently inserted into a plasmid that does function as a replicon in *C. crescentus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions: Strains used in these studies are listed in Table 1. *C. crescentus* strains are derived from CB15 (POINDEXTER 1964). *Escherichia coli* strains are all K-12 derivatives and *Klebsiella aerogenes* strains are derived from W70 (MACPHEE, SUTHERLAND and WILKINSON 1969). *C. crescentus* cultures were grown at 30° in PYE supplemented with 0.5 mM $CaCl_2$ or in M2-glucose minimal medium (JOHNSON and ELY 1977) that was modified by reducing the PO_4 concentration to 0.4 mM and adding imidazole HCl to 20 mM. *E. coli* and *K. aerogenes* strains were grown at 30° in LB or in W salts minimal medium (BENDER *et al.* 1977).

Matings: Fresh, broth grown cultures were grown (in the presence of antibiotics when appropriate) to densities of about 2×10^9/ml for *C. crescentus* or 5×10^9/ml for *E. coli* or *K. aerogenes*. To initiate mating, 0.5 ml of donor was mixed with 0.5 ml of recipient and 0.9 ml of the mixture was immediately collected on a Millipore filter (0.45 μm pore). The filter was aseptically transferred to an incubation plate (PYE when *C. crescentus* was one of the mating partners; LB when it was not) and incubated for 3 hr at 30°. At the end of the incubation the filter was transferred to 5 ml of CD (1 gm tryptone in 1 liter of water) and vortexed vigorously to resuspend the cells and terminate the mating. The following selection plates were used: PYE supplemented with 10 μg/ml of naladixic acid allowed growth of *C. crescentus* but not *E. coli*; LB allowed growth of *E. coli* and *K. aerogenes* but not of *C. crescentus*; W minimal salts with sodium citrate as sole carbon source allowed the growth of *K. aerogenes* but not *E. coli*. To select for transfer of drug resistance associated with the plasmid, kanamycin was added at 25 μg/ml and ampicillin was added at 25 μg/ml or 50 μg/ml. Tetracycline was added at 15 μg/ml when Tc^R *E. coli* or *K. aerogenes* were sought and at 0.5 μg/ml or 1.0 μg/ml when Tc^R *C. crescentus* were sought.

Physical detection of covalently closed circles: Strains CM 5000 and CM 5233 were grown in 5-ml cultures at 30° from approximately 10^7 cells/ml to approximately 5×10^8 cells/ml in low phosphate M2 medium. The CM 5000 culture was supplemented with adenine sulfate at 270 μM and with 10 μCi/ml of [^{3}H]adenine (14.1 Ci/mmol) throughout the growth period. The CM5233 culture was supplemented with 4 μCi/ml $^{32}PO_4$. Cell cultures were harvested by centrifugation. The pellets were resuspended in 0.5 ml of 25% sucrose in TES (CLEWELL 1972) and combined before lysis. Lysozyme (0.1 ml of a 5 mg/ml solution in TES) and disodium ethylenediaminetetraacetic acid (EDTA, 0.2 ml of a 0.25 M solution) were added to the mixed cells. After a 10-min incubation at room temperature, sodium dodecyl sulfate (SDS, 0.2 ml of a 10% solution) was added and the lysate was then sheared by pulling through an 18-gauge syringe needle approximately 20 times. The lysate was added to a Beckman 5.1-ml "quick seal" tube containing 3.5 ml of CsCl-saturated TES and 70 μl of ethidium bromide (10 mg/ml in TES). Centrifugation was performed in a VTi-65 vertical tube rotor at 289,000 × *g* for 15 hr. Most of the liquid portion of the gradient was removed from this tube with a syringe and put into another quick seal tube. Any solid material at the top or bottom of the gradient was left behind. Centrifugation was repeated, this time for 20 hr. This gradient was fractionated from the bottom. Portions (50 μl) of each 100-μl fraction were digested for 2 hr at 55° in 0.25 N KOH. Acid precipitable material was collected on Enzofilter glass fiber filters and the amount of radioactivity in this material was determined by scintillation counting, using commercially available scintillant.

RESULTS

Physical detection of RP4: As part of a study of DNA replication in *Caulobacter crescentus*, an analysis of the regulation of two plasmid replicons (RP4 and ColE1) was begun. The P-type drug-resistance plasmids, RP4 and RK2, were readily transferred from *E. coli* to *C. crescentus* (Table 2, line 0), confirming

TABLE 1

List of strains and plasmids used

Strain	Genotype	Source
Escherichia coli		
DB1287-1	*rpsL* (pRZ102)	D. BERG
DB1320-5	+, (pRZ112/F$^+$)	D. BERG
EB424	r$^-$m$^+$, *rpsL594*, *gal*, *lac*, *glk* (RP4)	This work
EB531	r$^-$m$^+$, *rpsL594*, *gal*, *lac*, *glk*	EG47 (GOLDBERG, BENDER and STREICHER 1974)
EB761	*rpsL*, (pRZ102, pVS1)	NC9413 → DB1287-1[a]
EB762	+(pRZ112, pVS1, F$^+$)	NC9413 → DB1320-5
EB812	r$^-$m$^+$, *rpsL594*, *gal*, *lac*, *glk*	This work[b]
EB813	r$^-$m$^+$, *rpsL594*, *gal*, *lac*, *glk* (pRK2013)	This work
EB814	r$^-$m$^+$, *rpsL594*, *gal*, *lac*, *glk* (pRK21)	This work
EB820	r$^-$m$^+$, *rpsL594*, *gal*, *lac*, *glk* (pRZ102)	Transformation[c]
EB821	r$^-$m$^+$, *rpsL594*, *gal*, *lac*, *glk* (pRZ112)	Transformation
EB824	r$^-$m$^+$, *rpsL594*, *gal*, *lac*, *glk* (pRZ102, pVS1)	NC9413 → EB820
EB825	r$^-$m$^+$, *rpsL594*, *gal*, *lac*, *glk* (pRZ112, pVS1)	NC9413 → EB821
NC9413	*pro*, *met* (pVS1)	(BARRETT *et al.* 1982)
RH202	r$^-$m$^-$, *lacY*, *thi*	(ADAMS *et al.* 1979)
Klebsiella aerogenes		
MK9000	*hutC515*, P1^S	(STREICHER, BENDER and MAGASANIK 1975)
Caulobacter crescentus		
CM5000	+(= CB15)	(POINDEXTER 1964)
CM5233	+(RP4)	This work
Plasmids		
pRZ102	ColE1::Tn5(Km^R)	(JORGENSEN, ROTHSTEIN and REZNIKOFF 1979)
pRZ112	ColE1::Tn5-112 (Km^R)	(JORGENSEN, ROTHSTEIN and REZNIKOFF 1979)
RP4	(Km^R Tc^R Ap^R)	H. MEADE
pVS1	RP4 Ω[kan](Km^S Tc^R Ap^R)	(BARRETT *et al.* 1982)
RK2	(Km^R Tc^R Ap^R)	D. HELINSKI
pRK21	ColE1::RK2 (Km^R Tc^R Ap^R)	(FIGURSKI, MEYER and HELINSKI 1979)
pRK2013	ColE1::RK2 *tra*$^+$ *ori*$^-$ (Km^R)	(FIGURSKI and HELINSKI 1979)

[a] The arrow indicates conjugal transfer from strain NC9413 to strain DB1287-1.

[b] Plasmids RK2, pRK2013 and pRK21 were transferred to the EB531 background by conjugation with K. *aerogenes* strains bearing them.

[c] Strains EB820 and EB821 arose after transformation with plasmid DNA purified from strains EB761 and 762, respectively. Low concentrations of DNA were used to reduce the possibility of double transforming events.

previous reports (ELY 1979; ALEXANDER and JOLLICK 1977). RP4 is completely stable in such conjugal transfers and we have never observed segregation of the three drug-resistance markers associated with the plasmid from each other (<0.1%), whether the transfer was from *E. coli* to *C. crescentus* or *vice versa*. The RP4 plasmid markers are maintained stably in *C. crescentus*, and segregants lacking RP4 are not generally observed after a single overnight growth, even under nonselective conditions (<0.1%). *C. crescentus* strains carrying RP4 were labeled with $^{32}PO_4$ and analyzed for the presence of covalently closed circular (CCC) DNA by cesium chloride equilibrium centrifugation in the presence of ethidium bromide (see MATERIALS AND METHODS). A *C. crescentus* strain carrying the antibiotic resistances characteristic of RP4 showed a peak of radioactivity in the region corresponding to CCC-DNA (Figure 1) whereas the parent strain (labeled with ^{3}H adenine), lacking the RP4 antibiotic resistances, showed no such peak. Fractions from the region of the gradient where CCC-DNA would lie were also analyzed by electron microscopy and both supercoiled DNA and circular DNA more than five times larger than the reference SV40 circles were seen (data not shown). These data demonstrate the physical presence of RP4 as an independent CCC-DNA molecule in *C. crescentus*.

Since the DNA in such gradients is radioactively labeled, it is possible to

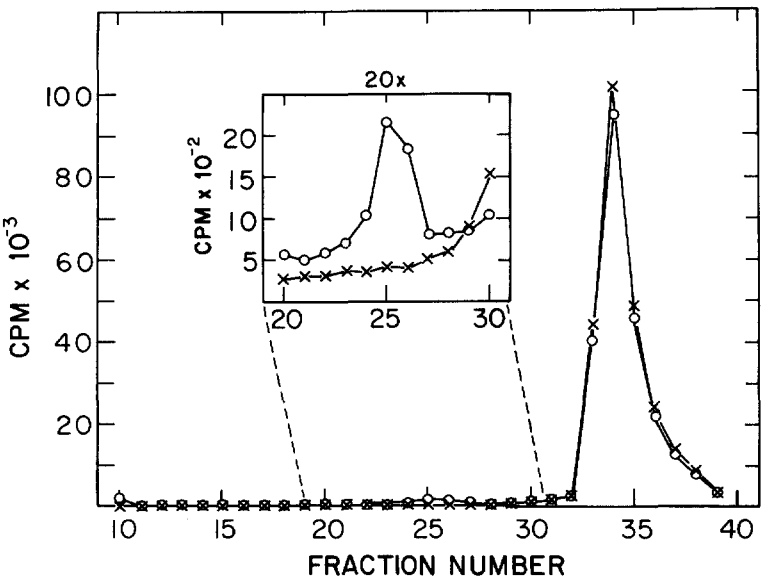

FIGURE 1.—Dye-buoyant density centrifugation of *C. crescentus* lysates. Strain CM5000, which exhibits no RP4 antibiotic resistance markers, was grown in the presence of ^{3}H-adenine for approximately six generations. Strain CM5233, which does exhibit appropriate RP4 antibiotic resistances, was grown in the presence of $^{32}PO_4$ for the same length of time. These two cultures were then mixed. Cell lysis and centrifugation were as described in MATERIALS AND METHODS. Fractions (0.1 ml) were collected from the region of the gradient where DNA was visually observed. A portion of each fraction (0.05 ml) was treated as described in MATERIALS AND METHODS. The inset is a 20-fold magnification of the region that would contain covalently closed circular DNA. ×, [^{3}H]; ○, [^{32}P].

compare the amount of radioactivity in the CCC-DNA to the total DNA and thus derive a minimal estimate of the number of copies of the plasmid per chromosome. In experiments like those shown in Figure 1, we obtained a minimum copy number of RP4 of 1.7 in *C. crescentus*. It must be emphasized that these numbers are underestimates of the true values since the large size of the plasmid leads to nicking and breakage of some plasmid molecules. Nevertheless, these data suggest that the copy number of RP4 in *C. crescentus* is low, as is found for RP4 in *E. coli*.

Nonmobilization of ColE1-derived plasmids: Several difficulties which arose when we attempted to repeat these experiments using ColE1 instead of RP4 prompted us to reexamine the question of whether the ColE1 replicon is functional in *C. crescentus*. In the first experiments, the plasmid pRZ102 was used. pRZ102 is a ColE1 plasmid carrying a kanamycin-resistance marker by virtue of the transposon Tn5 inserted into it (JORGENSEN, ROTHSTEIN and REZNIKOFF 1979). ELY (1979) reported that a Km^S derivative of RP4 could mobilize pRZ102 from *E. coli* to *C. crescentus*. In our experiment pVS1 (BARRETT *et al.* 1982) a nonreverting kanamycin-sensitive derivative of RP4, was used as the mobilizing plasmid. As reported by ELY (1979), Km^R transconjugants of a cross between strains EB824 (*E. coli* [pRZ102, pVS1]) and CM5000 (*C. crescentus* wild type) arose at a frequency of about 10^{-6} (Table 2, line 1), suggesting that the Km^R determinant from pRZ102 had been transferred from *E. coli* to *C. crescentus*. Four transconjugants from this cross were analyzed further.

The Km^R phenotype of all four strains was stable under selective and nonselective conditions. However, the Km^R determinant from pRZ102 showed tight genetic linkage to pVS1 markers whereas the ColE1 *imm* marker showed no such linkage. Three of the transconjugant strains, CM5220, CM5223 and CM5224, transferred the Km^R back to *E. coli* at very high frequency. The linkage of Km^R to pVS1 markers (Ap^R and Tc^R) was found to be nearly 100% (Table 2, lines 2, 4 and 5) suggesting that Km^R had become covalently associated with the pVS1 plasmid. In contrast, the ColE1 *imm* marker associated with pRZ102 was not detected in any of the backcross transconjugant strains EB829, EB830, and EB831, (Table 2, lines 2, 4 and 5). When these three *E. coli* backcross strains were mated further with wild type *C. crescentus* (Table 2, lines 6, 7 and 8) Km^R transfer was as frequent as pVS1 transfer, in marked contrast to the original mating with strain EB824 as donor, thus verifying a heritable alteration in the plasmids. When *C. crescentus* strains derived from these three crosses (with strains EB829, EB830 and EB831 as donors) were grown overnight under nonselective (drug-free) conditions, no Km^S segregants were detected (Table 2, lines 6, 7 and 8). Thus when Km^R was transferred from strain EB824 to CM5000 the Km^R marker became 100% linked genetically to pVS1 markers and became unlinked to the ColE1 *imm* marker of pRZ102, and this change was stably inherited through subsequent backcrosses. These results are easily explained if pVS1 acquired Km^R by transposition of Tn5 from pRZ102 to pVS1. The fourth transconjugant, strain CM5222, is resistant to *Km*, *Ap*, and *Tc* but is incapable of transferring any of these markers, even *Ap*, at the expected frequency. This suggests that the *tra* genes of pVS1 may have been mutated, perhaps by Tn5

TABLE 2

Transfer of the kanamycin-resistance determinant (Tn5) from pRZ102 between E. coli *and* C. crescentus

Donor	Recipient	Frequency of transconjugants			Transconjugants saved	
E. coli	*C. crescentus*	Tc^R	$(Km^R)^a$	Km^R/Tc^R	*C. crescentus*	
0. EB424	CM5000	8×10^{-1}	6×10^{-1}	10/10	CM5064	
1. EB824	CM5000	7×10^{-1}	6×10^{-6}	0/10	CM5220	
					CM5222	
					CM5223	
					CM5224	
C. crescentus	*E. coli*	Ap^R	(Km^R)	Km^R/Ap^R Km^R/Tc^R	*E. coli*	ColE1 phenotype of transconjugant
2. CM5220	EB531	7×10^{-1}	(2×10^{-1})	34/35 35/35	EB829	Sens.[b]
3. CM5222	EB531	$<10^{-3}$	$(<10^{-3})$	— —	—	—
4. CM5223	EB531	4×10^{-1}	(1×10^{-1})	30/30 30/30	EB830	Sens.
5. CM5224	EB531	9×10^{-1}	(1×10^{-1})	28/28 28/28	EB831	Sens.
E. coli	*C. crescentus*	Km^R	(Km^R)	Km^R/Total	*C. crescentus*	Segregation of Km^{R^c} from transconjugant
6. EB829	CM5000	8×10^{-1}	(9×10^{-2})	34/45	CMX29	<1%
7. EB830	CM5000	4×10^{-1}	(1×10^{-1})	12/30	CMX30	<1%
8. EB831	CM5000	8×10^{-1}	(3×10^{-2})	41/50	CMX31	<1%

Matings between *E. coli* and *C. crescentus* strains were performed on nitrocellulose filters as described in MATERIALS AND METHODS. The frequency of drug-resistant transconjugants is given per surviving recipient cell. For tetracycline resistance (Tc^R) and ampicillin resistance (Ap^R) these numbers are by direct count on antibiotic-supplemented selective medium.

[a] The efficiency of plating of kanamycin-resistant cells on kanamycin-supplemented selective medium is less than 1.0, so frequencies determined from direct count are given in parentheses. Frequency of Km^R exconjugants was also calculated by testing several surviving recipient cells for kanamycin resistance. Where determined, these values are given without parentheses.

[b] Sens., sensitive to colicin E1.

[c] A culture was grown from a single colony to saturation in antibiotic-free broth. One hundred colonies grown from this culture were tested for the presence of Km^R cells.

insertion, although other explanations are possible. Thus none of the transconjugants resulted from the transfer of pRZ102 to *C. crescentus* and its maintenance there.

Rearrangements in the absence of Tn5 transposase: To avoid Tn5 transposition, these experiments were repeated using pRZ112, a derivative of pRZ102 lacking the transposase gene of Tn5, but retaining Km^R (JORGENSEN, ROTHSTEIN and REZNIKOFF 1979), with pVS1 again used as the mobilizing plasmid. When strain EB825 (*E. coli* [pRZ112, pVS1]) was mated with strain CM5000 (wild-type *C. crescentus*), Km^R *C. crescentus* transconjugants again arose at a frequency of about 10^{-6} (Table 3, line 1). Eight transconjugants from this cross were backcrossed to *E. coli* strain EB531. The Km^R was again found to be nearly 100% linked to a pVS1 marker (Ap^R) (Table 3, lines 2–9) and Km^R was conjugally transferred to EB531 at a frequency characteristic of pVS1 transfer. This suggested that the Km^R marker from pRZ112 had become covalently associated with pVS1. Analysis of the transconjugants from this mating showed them to be of two distinct classes, based on linkage of the plasmid markers. One class (Table 3, lines 4 and 6) showed very stable linkage of Km^R, Tc^R and Ap^R even in the absence of selection (Table 3, lines 13, 14) but no association with the ColE1 *imm* marker of pRZ112. This result would be consistent with a transposition of the Km^R determinant (Tn5-112) from pRZ112 to pVS1 similar to that seen with Tn5 in Table 2. The second class of transconjugants (Table 3, lines 2, 3 and 7–9) showed stable linkage between Km^R, Ap^R and ColE1 *imm* but linkage of these markers to Tc^R was very unstable in the absence of selection (Table 3, lines 10, 11, 14 and 15). The Ap^R marker of pVS1 is itself a transposon (HEDGES and JACOB 1974) and the results with group two might be explained by transposition of Ap^R to pRZ112. To test this possibility plasmid DNA was prepared from strain EB832 and was used to transform RH202 selecting for Km^R at low DNA concentration to prevent cotransformation with two or more plasmids. Each of nine Km^R transformants so obtained was also Ap^R and ColE1 *imm*, but none were Tc^R, indicating that there had been a transposition of the Ap^R marker from pVS1 to pRZ112.

Effects of chimeric RK2::ColE1 plasmids: The analysis of the transconjugants described above demonstrated that the ColE1 replicon was not present independent of the RP4 replicon in any of the 12 strains analyzed in Tables 2 and 3. This might reflect either the inability of ColE1 to be transferred to *C. crescentus* or the inability of ColE1 to be maintained by *C. crescentus*. To distinguish between transfer and maintenance we tested conjugal transfer of two chimeric plasmids, pRK21 and pRK2013, from *E. coli* to *C. crescentus*. Plasmid pRK21 carries ColE1 covalently inserted into RK2; pRK2013 carries all of ColE1 and all the transfer genes of RK2 but lacks the replication origin of RK2 (FIGURSKI and HELINSKI 1979). Both plasmids were transferred to *K. aerogenes* recipients as efficiently as RK2 confirming that the transfer functions were intact (Table 4, lines 1–3). Plasmid pRK21 was transferred to *C. crescentus* with about the same efficiency as RK2 (Table 4, lines 4 and 6) but pRK2013 gave no kanamycin-resistant *C. crescentus* transconjugants ($<10^{-8}$), thus showing that the ColE1 replicon was not sufficient for maintenance in *C. crescentus*. Furthermore, the

TABLE 3

Transfer of the kanamycin-resistance determinant (Tn5-112) from pRZ112 between E. coli *and* C. crescentus

Donor	Recipient	Frequency of transconjugants				Transconjugants saved	
E. coli	*C. crescentus*	Tc^R	(Km^R)	Km^R/Tc^R		*C. crescentus*	
1. EB825	CM5000	7×10^{-1}	(6×10^{-6})	0/10		CM5225 through CM5232	
C. crescentus	*E. coli*	Ap^R	(Km^R)	Km^R/Ap^R	Tc^R/Ap^R	*E. coli*	ColE1 phenotype of transconjugant
2. CM5225	EB531	9×10^{-1}	(2×10^{-1})	31/31	29/31	EB832	Imm.[a]
3. CM5226	EB531	8×10^{-1}	(4×10^{-1})	21/21	20/21	EB833	Imm.
4. CM5227	EB531	1×10^{-1}	(1×10^{-1})	9/9	9/9	EB834	Sens.[b]
5. CM5228	EB531	6×10^{-1}	(6×10^{-1})	31/31	30/31	EB835	N.D.[c]
6. CM5229	EB531	5×10^{-1}	(3×10^{-1})	30/31	29/31	EB836	Sens.
7. CM5230	EB531	4×10^{-2}	(4×10^{-2})	2/2	2/2	EB837	Imm.
8. CM5231	EB531	9×10^{-1}	(4×10^{-1})	20/20	15/20	EB838	Imm.
9. CM5232	EB531	8×10^{-1}	(2×10^{-1})	22/22	18/22	EB839	N.D.
E. coli	*C. crescentus*	Km^R	(Km^R)	Km/total		Km^R transconjugant saved C. crescentus	Segregation of Km^R from transconjugants
10. EB832	CM5000	$<10^{-1}$	(3×10^{-4})	0/16		CMX32	19%
11. EB833	CM5000	$<10^{-1}$	(6×10^{-4})	0/22		CMX33	27%
12. EB834	CM5000	7×10^{-1}	(2×10^{-1})	34/50		CMX34	0%
13. EB836	CM5000	6×10^{-1}	(1×10^{-1})	20/32		CMX36	0%
14. EB837	CM5000	$<10^{-1}$	(8×10^{-4})	0/31		CMX37	35%
15. EB838	CM5000	$<10^{-1}$	(7×10^{-4})	0/18		CMX38	42%

[a] Immune to colicin E1.
[b] Sensitive to colicin E1.
[c] Not determined.

TABLE 4

Transfer of chimeric plasmids from E. coli *to* K. aerogenes *(strain MK9000) and* C. crescentus *(strain CM5000)*

	Plasmid	Donor strain	Recipient strain	Frequency Km^R
1	RK2	EB812	MK9000	3×10^{-1}
2	pRK2013	EB813	MK9000	3×10^{-1}
3	pRK21	EB814	MK9000	3×10^{-1}
4	RK2	EB812	CM5000	1×10^{-1}
5	pRK2013	EB813	CM5000	$<10^{-8}$
6	pRK21	EB814	CM5000	3×10^{-1}

Donor and recipient strains were mated on nitrocellulose filters as described in MATERIALS AND METHODS and the conjugation mixture was plated on selective medium with and without kanamycin. Frequencies are given per recipient. Selective medium for experiments 1 to 3 contained sodium citrate as sole carbon source (which *E. coli* cannot use); for experiments 4 to 6 selective medium was PYE agar supplemented with 10 μg/ml naladixic acid (to which *E. coli*, but not *C. crescentus* is sensitive).

stable maintenance of pRK21 showed that the failure of the ColE1 replicon was not due to any "suicide function" present on ColE1.

Since the ColE1 sequences had been inserted into pRK21 after cleavage with the restriction endonuclease *Eco*RI, these sequences differ from those of native ColE1 in that ColE1 encodes the production of colicin E1 whereas pRK21 does not. However "colicin suicide" cannot be invoked to explain the failure of pRZ102 and pRZ112 to be maintained since we have confirmed that the Tn5 insertion abolished the ability of pRZ102 and pRZ112 to produce colicin E1 (D. BERG, cited by JORGENSEN, ROTHSTEIN and REZNIKOFF 1979). Furthermore, both pRZ102 and pRZ112 retain their immunity to colicin E1. Thus ColE1 sequences can be maintained in *C. crescentus*, but only when covalently linked to another replicon.

DISCUSSION

The data presented here demonstrate that the ColE1 replicon does not function in *C. crescentus*. Whenever ColE1 sequences were stably maintained in *C. crescentus* they were found to be covalently attached to another replicon that could be used by *C. crescentus* (in this instance the RP4 or RK2 replicon). The transfer of a ColE1 marker (Tn5) had previously been interpreted as suggesting that ColE1 could be maintained in *C. crescentus* (ELY 1979). A further analysis of these Km^R transconjugants has shown that the Km^R transfer resulted either from transposition or cointegration events.

The data in Table 2 argue strongly that the transfer of kanamycin-resistance from *E. coli* strain EB824 (pVS1, pRZ102) resulted from a transposition of Tn5 from pRZ102, which cannot be maintained by *C. crescentus*, to the fertile plasmid pVS1 which can be maintained. The resulting pVS1::Tn5 was stable and freely transmissible to *C. crescentus*. These events are fully consistent with the known transposition activities of Tn5 (D. BERG 1977).

The data in Table 3 can likewise be explained by transposition events. The

transposition of Tn*3* from pVS1 to pRZ112 was demonstrated in strain EB832 and likely for strains EB833, EB837 and EB838. Tn*3* transposition involves as a required intermediate a cointegrate plasmid containing two copies of the Tn*3* transposon flanking single copies of the two plasmids. This cointegrate structure is unstable and resolution by site-specific recombination (HEFFRON *et al.* 1980) yields two independent plasmids each carrying one copy of Tn*3*. Transfer of such a cointegrate intermediate would carry the kanamycin-resistance determinant into *C. crescentus* and would explain the instability of the kanamycin resistance in the resulting transconjugants. Upon resolution (in *C. crescentus*) the kanamycin-resistance determinant would again be associated with only the ColE1-type replicon, pRZ112::Tn*3*, and hence be lost. In contrast, resolution of this unstable cointegrate in *E. coli* would result in stable maintenance of both replicons, pRZ112::Tn*3* and pVS1, and would explain the independent segregation of the Tc^R marker (carried on pVS1) and the Ap^R marker (carried on pRZ112::Tn*3* as well as pVS1) seen in Table 3, lines 2–9.

The unstable cointegrate would also explain the different transfer efficiency of the Km^R determinant from *C. crescentus* and *E. coli* donors (Table 3, lines 2 and 10). In *C. crescentus* only those cells where the cointegrate was still unresolved would be Km^R and therefore Km^R would transfer efficiently. In *E. coli*, resolution of the cointegrate would leave the cells Km^R because the pRZ112 plasmid is quite stable in *E. coli*. Mating of Km^R would then require a mobilization of the pRZ112 plasmid by pVS1—an event almost as rare as the initial mating with these two plasmids (Table 3, line 1).

The group one strains from Table 3 (EB834 and EB836) were unexpected and appear to have resulted from a transposition of Tn*5*-112 from pRZ112 to pVS1, similar to the transposition of Tn*5* from pRZ102 seen in Table 2. The Tn*5*-112 is deleted for the functional transposase gene, located in the right inverted repeat. The left inverted repeat contains a copy of the transposase gene but its activity is abolished because of an ochre codon near the carboxyl terminus (ROTHSTEIN and REZNIKOFF 1981). Low level ochre suppression would allow occasional transposition, but we purposely chose the *E. coli* su^- strain EB531 for these experiments because of its *rpsL594* allele which greatly reduces ribosomal misreading of nonsense codons (FRIEDMAN and YARMOLINSKY 1972; R. A. BENDER and D. BOTSTEIN, unpublished results). Although strains EB834 and EB836 might have been formed by a rare transposition of Tn*5*-112, it is conceivable that they resulted from a cointegrate (as in group two strains) followed by deletion of one of the Ap^R markers and some of the pRZ112 sequences including the ColE1 *imm* marker. We have not yet distinguished between the two models of Tn*5*-112 transposition and cointegration-deletion.

The data previously interpreted as suggesting independent maintenance of pRZ102 in *C. crescentus* may not be inconsistent with our finding that the ColE1 replicon cannot be maintained in *C. crescentus*. Independent maintenance was proposed on the basis of three key observations: 1) independent segregation of Tc^R and Km^R markers from *C. crescentus* transconjugants, 2) stability of Km^R in the absence of the RP4 markers, and 3) low frequency of Km^R transfer of *E. coli* in a backcross. If loss of Km^R occurred by resolution of a cointegrate and retention of Km^R occurred by transposition of Tn*5* into the chromosome, then

the first two observations are consistent with our data. The RP4 *kan*$^-$ plasmid used by ELY reverts to Km^R, in contrast to pVS1 (B. ELY, personal communication); hence the rare Km^R *E. coli* transconjugants may carry a Km^R derivative of RP4 *kan*$^-$. Thus simple assumptions would resolve the apparent contradictions.

The inactivity of the ColE1 replicon in *C. crescentus* frustrates the hope of using ColE1-derived recombinant-DNA plasmids for complementation in *C. crescentus* and makes ColE1-derived plasmids unsuitable for finding conditions that will make *C. crescentus* competent for transformation. However, our results demonstrate the importance of plasmids like pRK2013 which can be replicated in *E. coli* and transferred to *C. crescentus* but cannot be maintained in *C. crescentus*. Such plasmids are ideal vectors for transposon mutagenesis and since their loss is passive rather than "suicidal" (as seen with RP4::Mu*cts*62), there are none of the problems associated with DNA rearrangements other than those mediated by the transposon under study. Plasmids like pRK2013 are also ideal agents for mobilizing plasmids like R1162 or miniRP4 to *C. crescentus* (DITTA *et al.* 1980). Since pRK2013 cannot be maintained in *C. crescentus*, transfer of the mobilized plasmid alone will occur and the potential presence of the mobilizing plasmid can be eliminated. Finally, plasmids like pRK2013 are ideal vectors for the isolation of origins of replication from organisms like *C. crescentus*, where the activity in *E. coli* of such an origin of replication cannot be presupposed. The ColE1 origin provides replication function in *E. coli* so that pieces of *C. crescentus* DNA can be isolated by recombinant DNA techniques. Only those plasmids that carry a *C. crescentus* replication origin will be stably maintained upon transfer to *C. crescentus*.

We thank D. HELINSKI, D. BERG and B. ELY for providing strains and D. BERG and B. ELY for critical reading of the manuscript. This work was supported by grants from the Public Health Service, National Institutes of Health (AI15822 and GM27111). E.O.N. was supported by an NIH predoctoral training grant (GM07315). R.A.B. was recipient of Junior Faculty Research Award from the American Cancer Society (JFRA-3).

LITERATURE CITED

ADAMS, J., T. KINNEY, S. THOMPSON, L. RUBIN and R. B. HELLING, 1979 Frequency-dependent selection for plasmid-containing cells of *Escherichia coli*. Genetics **91:** 627–637.

ALEXANDER, J. L. and J. D. JOLLICK, 1977 Transfer and expression of *Pseudomonas* plasmid RP1 in *Caulobacter*. J. Gen. Microbiol. **99:** 325–331.

BARRETT, J. T., R. H. CROFT, D. M. FERBER, C. J. GERARDOT, P. V. SCHOENLEIN and B. ELY, 1982 Genetic mapping with Tn5-derived auxotrophs of *Caulobacter crescentus*. J. Bacteriol. **151:** 888–898.

BENDER, R. A., 1981 An improved generalized transducing phage for *Caulobacter crescentus*. J. Bacteriol. **148:** 734–735.

BENDER, R. A., N. AGABIAN and L. SHAPIRO, 1980 Cell differentiation in *Caulobacter crescentus*. pp. 17–39. In: *The Molecular Genetics of Development*. Edited by T. LEIGHTON and W. F. LOOMIS. Academic Press, New York.

BENDER, R. A., K. A. JANSSEN, A. D. RESNICK, M. BLUMENBERG, F. FOOR and B. MAGASANIK, 1977 Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*. J. Bacteriol. **129:** 1001–1009.

BERG, D. E., 1977 Insertion and excision of the kanamycin resistance determinant Tn5. pp.

205–212. In: *DNA Insertion Elements, Plasmids, and Episomes.* Edited by A. I. BUKHARI, J. A. SHAPIRO and S. L. ADHYA. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

CLEWELL, D. B., 1972 Nature of ColE1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110:** 667–676.

DEGNAN, S. T. and A. NEWTON, 1972 Chromosome replication during development in *Caulobacter crescentus*. J. Mol. Biol. **64:** 671–680.

DITTA, G., S. STANFIELD, D. CORBIN and D. R. HELINSKI, 1980 Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:** 7347–7351.

ELY, B., 1979 Transfer of drug resistance factors to the dimorphic bacterium *Caulobacter crescentus*. Genetics **91:** 371–380.

FIGURSKI, D. H. and D. R. HELINSKI, 1979 Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:** 1648–1652.

FIGURSKI, D. H., R. J. MEYER and D. R. HELINSKI, 1979 Suppression of ColE1 replication properties by the Inc P-1 plasmid RK2 in hybrid plasmids constructed *in vitro*. J. Mol. Biol. **133:** 295–318.

FRIEDMAN, D. I. and M. B. YARMOLINSKY, 1972 Prevention of the lethality of induced λ prophage by an isogenic λ plasmid. Virology **50:** 472–482.

GOLDBERG, R. B., R. A. BENDER and S. L. STREICHER, 1974 Direct selection for P1 sensitive mutants of enteric bacteria. J. Bacteriol. **118:** 810–814.

HEDGES, R. W. and A. E. JACOB, 1974 Transposition of ampicillin resistance from RP4 to other replicons. Mol. Gen. Genet. **132:** 31–40.

HEFFRON, F., R. KOSTRIKEN, C. MORITA and R. PARKER, 1980 Tn*3* encodes a site-specific recombination system: identification of essential sequences, genes and the actual site of recombination. Cold Spring Harbor Symp. Quant. Biol. **45:** 259–268.

JOHNSON, R. C. and B. ELY, 1977 Isolation of spontaneously-derived mutants from *Caulobacter crescentus*. Genetics **86:** 25–32.

JORGENSEN, R. A., S. J. ROTHSTEIN and W. S. REZNIKOFF, 1979 A restriction enzyme cleavage map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177:** 65–72.

MACPHEE, D. G., I. W. SUTHERLAND and J. T. WILKINSON, 1969 Transduction in *Klebsiella*. Nature **221:** 475–476.

OSLEY, M. A. and A. NEWTON, 1978 Regulation of cell cycle events in asymmetrically dividing cells: functions required for DNA initiation and chain elongation in *Caulobacter crescentus*. J. Bacteriol. **135:** 10–17.

POINDEXTER, J., 1964 Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. **28:** 231–295.

ROTHSTEIN, S. J. and W. S. REZNIKOFF, 1981 The functional differences in the inverted repeats of Tn*5* are caused by a single base pair nonhomology. Cell **23:** 191–199.

SHAPIRO, L., 1976 Differentiation in the *Caulobacter* cell cycle. Annu. Rev. Microbiol. **30:** 377–407.

SHEFFERY, M. and A. NEWTON, 1981 Regulation of periodic protein synthesis in the cell cycle: control of initiation and termination of flagellar gene expression. Cell **24:** 49–57.

STREICHER, S. L., R. A. BENDER and B. MAGASANIK, 1975 Genetic control of glutamine synthetase in *Klebsiella aerogenes*. J. Bacteriol. **121:** 320–331.

Corresponding editor: D. SCHLESSINGER