Transfer and Expression of *Pseudomonas* Plasmid RP1 in *Caulobacter*

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SUMMARY

This study demonstrates that the host range of *Pseudomonas* plasmid RP1 includes the genus *Caulobacter*. *Caulobacter* was shown to acquire three antibiotic resistance markers located in RP1. A fourth plasmid marker, susceptibility to an RNA bacteriophage, was not expressed, but could be transferred from *Caulobacter* to *Escherichia coli*. The lack of phenotypic expression of the phage marker was manifested by the inability of the phage to adsorb or to produce plaques on *Caulobacter* transipients.

Matings of *Pseudomonas aeruginosa* and *Caulobacter vibrioides* cv6 were carried out in the presence of bacteriophage $\phi 6$, a DNA phage that infects and kills only swarmer cells of *Caulobacter*. No decrease in plasmid transfer in the presence of phage $\phi 6$ was detected, suggesting that stalked cells, and not swarmer cells, serve as recipients.

Our evidence suggests that transfer of chromosomal segments from *Caulobacter* may be mediated by plasmid RP1; such segments are not stably maintained.

INTRODUCTION

Pseudomonas aeruginosa PAO67 harbours plasmid RP1, a covalently closed circular DNA molecule of molecular weight approximately 40 Mdaltons (Grinsted *et al.*, 1972). RP1 specifies resistance to carbenicillin, tetracycline and neomycin/kanamycin, and can be transferred to a variety of species representing related and unrelated genera (Olsen & Shipley, 1973). The host range of this plasmid includes Enterobacteriaceae, soil saprophytes, *Neisseria perflava* and photosynthetic bacteria. With the acquisition of drug resistance, these strains also become sensitive to a small RNA bacteriophage designated PRR1.

Because of the wide host range of RP1, we investigated the possibility that this plasmid can transfer into *Caulobacter*. Poindexter (1964) and Shapiro, Agabian-Keshishian & Bendis (1971) have shown that *Caulobacter* displays a sequence of well-defined developmental stages. Three types of cells have been identified during its life-cycle: (i) the motile swarmer cell which carries a single flagellum at one pole; (ii) the non-motile cell which has a polar outgrowth of cellular material known as the stalk; and (iii) the dividing cell which has a stalk at one pole and a flagellum at the other. Equatorial division yields the motile and sessile daughter cells. Shapiro & Maizel (1973) demonstrated that a subsequent morphological alteration occurs when the swarmer cell sheds its flagellum while synthesizing a new stalk at the same site, thereby effecting the morphogenesis of a swarmer to stalked cell.

Because of its diphasic life-cycle, *Caulobacter* is a good model system for studying

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Table 1. *Bacterial strains used in this study*

Strain	Characteristics	Source
<i>Pseudomonas aeruginosa</i> PA067	His ⁻ , FP ⁻ , PRR1-s, Car-r, Tet-r, Kan-r,	R. H. Olsen, University of Michigan
<i>Escherichia coli</i> CSH29	Trp ⁻ , B ₁ ⁻ , F ⁻ , PRR1-r, Car-s, Tet-s, Kan-s	E. M. Schervish-Swierkosz, Wayne State University
<i>Pseudomonas aeruginosa</i> ATCC17933	Prototroph, PRR1-r, Car-s, Tet-s, Kan-s	L. B. Schook, Wayne State University
<i>Caulobacter vibrioides</i> CV6	Prototroph, PRR1-r, Car-s, Tet-s, Kan-s	J. D. Jollick
<i>C. crescentus</i> WS12	Met ⁻ , PRR1-r, Car-r, Tet-s, Kan-s, Str-s	J. D. Jollick
<i>C. crescentus</i> WS30	Met ⁻ , PRR1-r, Car-r, Tet-s, Kan-s, Str-r	J. D. Jollick

Abbreviations used throughout this paper: Car, carbenicillin; Kan, Kanamycin; Str, streptomycin; Tet, tetracycline; s, sensitive to antibiotic or phage; r, resistant to antibiotic or phage.

bacterial differentiation. The introduction of plasmid RP1 into *Caulobacter*, which we have demonstrated in this investigation, will provide an additional way in which gene exchange and expression can be studied in this organism.

METHODS

Organisms. The bacterial strains used in this study are listed in Table 1. RNA bacteriophage PRR1 was obtained from Professor R. H. Olsen, Department of Microbiology, University of Michigan, Ann Arbor, U.S.A.

Media. The complete medium used for the growth of *Caulobacter*, *Escherichia coli* and *Pseudomonas aeruginosa* strains and for propagation of phages $\phi 6$ and PRR1 was the peptone yeast extract (PYE) medium of Poindexter (1964). The chemically defined (minimal) medium was described previously by Jollick & Tran (1975). For the growth of auxotrophic *E. coli* CSH29, minimal medium was supplemented with tryptophan ($40 \mu\text{g ml}^{-1}$) and thiamin ($5 \mu\text{g ml}^{-1}$). Auxotrophic *P. aeruginosa* PA067 was grown in minimal medium supplemented with histidine ($40 \mu\text{g ml}^{-1}$). Auxotrophic *Caulobacter crescentus* was grown in minimal medium supplemented with methionine ($40 \mu\text{g ml}^{-1}$). The selective medium was the appropriate minimal medium with the minimal inhibitory concentration (m.i.c.) of carbenicillin, tetracycline, streptomycin or kanamycin.

Antibiotic minimal inhibitory concentrations. Levels of susceptibility of donor and recipient strains to the various antibiotics were determined by inhibition of colony formation on the appropriate antibiotic minimal plates. Serial 10-fold dilutions of 18 h PYE broth cultures were plated on appropriate media. Colony counts were made after 48 h incubation for *P. aeruginosa* and *E. coli* (incubated at 37°C), and after 1 week for *Caulobacter* (incubated at 30°C). The m.i.c. was defined as the lowest concentration of antibiotic which inhibited visible colony formation at the end of the incubation. Recipient m.i.c. dictated the concentrations of antibiotics used in the selection procedure. The results are shown in Table 2.

Mating procedure. Donor and recipient cell titres were adjusted to approximately 3×10^8 colony-forming units (c.f.u.) ml^{-1} , mixed in PYE broth at a ratio of 1:1, and then incubated at the optimum growth temperature of the recipient (30°C for *Caulobacter*, 37°C for *E. coli* and *P. aeruginosa*) for 3 h. The mating mixture was then diluted and plated on the appropriate selective medium. Transcipient colonies were counted after 48 h when either *P. aeruginosa* or *E. coli* was the recipient, and after 2 weeks when *Caulobacter* was the recipient.

Table 2. Minimal inhibitory concentrations ($\mu\text{g ml}^{-1}$) of carbenicillin, kanamycin, tetracycline and streptomycin

Strain	Car	Kan	Tet	Str
<i>P. aeruginosa</i> PAO67	> 500	> 500	> 500	—
<i>P. aeruginosa</i> ATCC17933	30	150	15	—
<i>E. coli</i> CSH29	25	10	5	—
<i>C. vibrioides</i> CV6	500	10	1	5
<i>C. crescentus</i> WS12	500	200	1	—
<i>C. crescentus</i> WS30	500	200	1	200

Determination of phage PRR1 susceptibility of R⁺ transcipts. R⁺ transcipts of *P. aeruginosa* ATCC17933, *E. coli* CSH29, and *Caulobacter* strains CV6 and WS12 selected on the minimal antibiotic plates were tested for susceptibility to phage PRR1 by the agar layer method of Adams (1959). PYE agar was used as the base layer. In all cases 0.2 ml of a 24 h PYE broth culture was added to 3.0 ml of molten 0.75 % (w/v) agar. The molten agar was poured on the base and allowed to harden, and then single drops (0.01 ml) of serial 10-fold dilutions of phage PRR1 (1.88×10^9 plaque-forming units ml^{-1}) were placed on the plates. The plates were incubated at the appropriate temperature and examined at 24 and 48 h.

Determination of adsorption of phage PRR1 to R⁺ transcipts. Specific adsorption of phage PRR1 to R⁺ *Caulobacter* strains and *E. coli* R⁺ was determined by assaying unadsorbed phage in cell/phage mixtures. Phage PRR1, at a multiplicity of infection (m.o.i.) of 0.1, was added to an 18 h broth culture of the strain to be tested. After 20 min, a 0.1 ml sample of the mixture was removed, diluted 1:100 in PYE medium, and centrifuged at 10 000 g for 10 min to remove bacterial cells and infected centres. The supernatant fluid was then filtered (0.45 μm pore size Millipore filter), diluted and plated in soft agar overlays with *P. aeruginosa* PAO67 as the indicator strain. Unadsorbed phage was expressed as the percentage of input phage that remained free.

Determination of the effect of phage ϕ 6 on the formation of Caulobacter vibrioides CV6 R⁺ transcipts. Matings between *P. aeruginosa* PAO67 and *C. vibrioides* CV6 were performed as described above except that 1.0 ml phage ϕ 6 was added to give a m.o.i. of 20 phage per recipient at the time when the bacterial strains were mixed. A parallel mating to which 1.0 ml PYE broth was added instead of phage served as a control. After the 3 h incubation, the mixtures were diluted, plated on appropriate selective media and examined for transcipt formation.

Chromosomal marker transfer mediated by RP1. To determine if chromosomal marker transfer in *Caulobacter* could be mediated by RP1, an 18 h broth culture of strain CV6 R⁺ (which in the R⁻ state is not a donor) was mated with an 18 h broth culture of the auxotrophic strain WS30 which requires methionine. After 3 h the mating mixture was plated on selective media and also on minimal medium to test for methionine marker transfer alone. These plates were incubated at 30 °C for 2 weeks and then examined for transcipt colonies. Streptomycin at 5 $\mu\text{g ml}^{-1}$ was used to counterselect against the CV6 R⁺ donor.

RESULTS AND DISCUSSION

Mating results

Determinants for resistance to all three antibiotics were transferred from *P. aeruginosa* PAO67 to *E. coli* CSH29, *P. aeruginosa* ATCC17933 and to *C. vibrioides* CV6. The transfer yields (Table 3) were comparable in the *Pseudomonas* $\times$ *Pseudomonas* and the *Pseudomonas*

Table 3. *Yield of antibiotic-resistant transciipients with P. aeruginosa PAO67 as donor*

Results are expressed as the number of transciipients per donor.

Recipient	Kan-r	Car-r	Tet-r
<i>E. coli</i> CSH29	3.3×10^{-5}	1.2×10^{-4}	1.6×10^{-4}
<i>P. aeruginosa</i> ATCC17933	1.0×10^{-5}	1.9×10^{-4}	8.9×10^{-4}
<i>C. vibrioides</i> CV6	7.7×10^{-4}	5.3×10^{-4}	1.1×10^{-3}
<i>C. crescentus</i> WS12	4.4×10^{-4}	—	6.9×10^{-4}

× *E. coli* matings. The *Pseudomonas* × *C. vibrioides* CV6 mating seemed to be the most efficient. Anderson (1968) and Anderson & Threlfall (1970) have suggested several factors which may influence the frequency at which an R factor is transferred: (i) the identity of the plasmid and its state of repression in a given host; (ii) non-specific factors affecting transfer between mating pairs; and (iii) host-controlled modification and restriction systems. Olsen & Shipley (1973) have suggested that plasmid RP1 is derepressed on the basis of bacteriophage plating criteria and the failure of mixed R⁺/R⁻ donor cultures to elevate the frequency of transfer of the plasmid.

Differences between the numbers of transciipients resistant to the three antibiotics in a cross are likely to be due to differences in the time of expression for the markers or differences in actual transfer frequency and not to segregation, since resistant colonies selected on one antibiotic grew when transferred to selective media containing the other two antibiotics.

Susceptibility of R⁺ transciipients to phage PRR1

In each mating R⁺ transciipients were selected from each of the three antibiotic selection plates. Ten colonies from each plate were streaked for isolation on PYE medium to eliminate possible donor contamination. A PYE broth culture served as the inoculum for the agar overlays used to test for phage PRR1 susceptibility. None of the recipients were susceptible to phage PRR1 before mating with strain PAO67. *Escherichia coli* CSH29 and *P. aeruginosa* ATCC17933, upon acquisition of the plasmid, exhibited susceptibility to phage PRR1. However, *Caulobacter vibrioides* CV6 did not become susceptible to PRR1 as determined by spotting the phage lysate on lawns of CV6 R⁺ strains. In all *P. aeruginosa* ATCC17933 R⁺ and *E. coli* CSH29 R⁺ isolates, productive phage infection, as determined by plaque formation could be detected (Table 4). Since phage PRR1 susceptibility was not detected in CV6 R⁺ transciipients, either the phage marker was not being transferred, or perhaps not being expressed in *Caulobacter*. It was established that the phage susceptibility marker had been transferred to the CV6 R⁺ transciipient by further mating with *E. coli* CSH29. Strain CV6 R⁺ transferred the RP1 plasmid complete with antibiotic markers and phage susceptibility to *E. coli* CSH29 as efficiently as did *P. aeruginosa* PAO67. Twenty *E. coli* CSH29 R⁺ transciipient colonies from this cross selected from each antibiotic plate became susceptible and productive for phage PRR1 (Table 4). Therefore, the genetic marker for phage PRR1 susceptibility was present but not expressed in *Caulobacter*. Since *Caulobacter* R⁺ transciipients were able to transfer the phage marker, attempts were made to establish the step in the phage replicative cycle that was blocked. Failure of phage PRR1 susceptibility to be expressed in *Caulobacter* could result from a lack of receptor sites or a block of other parts of the replicative cycle. Adsorption tests of phage PRR1 were carried out with the R⁺ transciipients. *Pseudomonas aeruginosa* PAO67, the donor strain that was used for the

Table 4. Phage PRR1 susceptibility of transipients

Donor	Recipient (R ⁺ transipients)	Susceptible*	Productive†
<i>P. aeruginosa</i> PAO67	<i>E. coli</i> CSH29	+	+
<i>P. aeruginosa</i> PAO67	<i>P. aeruginosa</i> ATCC17933	+	+
<i>P. aeruginosa</i> PAO67	<i>C. vibrioides</i> cv6	—	—
<i>C. vibrioides</i> cv6 R ⁺	<i>E. coli</i> CSH29	+	+

* Determined by inhibition of bacterial growth in agar overlay.

† Determined by plaque formation in agar overlay.

Table 5. Effect of phage $\phi 6$ on *C. vibrioides* cv6 transipient formation

Results are expressed as the number of transipients per ml in the mating mixture.

Cross	Kan	Car	Tet
PAO67 $\times$ CV6	2.0×10^4	4.6×10^4	2.1×10^4
PAO67 $\times$ CV6 + $\phi 6$	1.8×10^4	5.6×10^4	3.2×10^4
PAO67 $\times$ CV6	1.32×10^5	3.4×10^4	2.5×10^4
PAO67 $\times$ CV6 + $\phi 6$	1.22×10^5	3.6×10^4	1.1×10^4
PAO67 $\times$ CV6	1.44×10^4	6.10×10^4	2.3×10^3
PAO67 $\times$ CV6 + $\phi 6$	1.48×10^4	5.74×10^4	1.0×10^3

propagation of phage PRR1, was also used as a positive control. *Caulobacter vibrioides* cv6 and *E. coli* CSH29 were used as negative controls. *Pseudomonas aeruginosa* PAO67 adsorbed 34 % of the input phage in 20 min, while the R⁻ strains of *C. vibrioides* cv6 and *E. coli* CSH29 showed no adsorption. With *C. vibrioides* cv6 R⁺, no adsorption of phage could be demonstrated, but *E. coli* CSH29 R⁺ showed 39 % adsorption. These results indicate that phage infection by PRR1 of *C. vibrioides* cv6 R⁺ must be inhibited at the adsorption stage.

It is not known if pili are absent from strain cv6 R⁺ or if cv6 R⁺ produces a defective pilus to which PRR1 is unable to adsorb. There is a possibility that in *Caulobacter* R⁺ cultures only a small fraction of cells are piliated at any one time, but this is not likely since we have demonstrated that strain cv6 R⁺ donates the plasmid, as efficiently as *P. aeruginosa* PAO67 in the case of transfer to *E. coli* CSH29 (unpublished results). It is therefore likely that pili are present; however, it is possible that in *Caulobacter* they are modified in such a way that they still function in mating but not in phage adsorption. It is also possible that the phage receptor pili and conjugal pili are different.

Effect of Caulobacter phage $\phi 6$ on the viability of C. vibrioides cv6 and the formation of cv6 R⁺ transipients

Caulobacter is one of the few bacteria which undergoes differentiation. Jollick (1972) and Jollick & Wright (1974) characterized DNA bacteriophage $\phi 6$ which has a wide host range and which adsorbs to and kills only the swarmer cells in *Caulobacter* cultures. It was of interest to learn what effect phage $\phi 6$ might have on R⁺ transipient formation since this would provide information on which type of cell participates in mating. If the swarmer cell was the recipient in the mating, then its elimination from the recipient population would reduce the number of transipients recovered. As a positive control, it was demonstrated that a reduction in the recipient population did reduce the number of transipients: a reduction of *C. vibrioides* cv6 from 3×10^8 cells ml⁻¹ to approximately 1.5×10^8 cells ml⁻¹ in mating mixtures led to a 50 % reduction in transipients ml⁻¹ recovered. It was also shown

Table 6. *Transfer of plasmid RP1 in the Caulobacter cross CV6 R⁺ × WS30*

Transcript selection medium*	No. of transcriptants ml ⁻¹	Marker transferred
Min + Met + Kan ₂₀₀ + Str ₅	4.67 × 10 ³	Kan
Min + Met + Tet ₅ + Str ₅	9.0 × 10 ¹	Tet
Min + Kan ₂₀₀ + Str ₅	4.46 × 10 ³	Kan + Met
Min + Tet ₅ + Str ₅	0	—
Min + Str ₅	1.52 × 10 ³	Met

* Min, minimal medium; Met, methionine. Subscripts indicate the concentration of antibiotic in µg ml⁻¹.

that phage $\phi 6$ at a m.o.i. of 20 reduced the viable count of strain CV6 cultures to about 12 % of the control. Phage $\phi 6$ had no effect on *P. aeruginosa* PAO67. The effect of phage $\phi 6$ on three different matings involving strain CV6 is presented in Table 5. Phage $\phi 6$ caused no apparent reduction in transcript formation. Therefore, the swarmer cell did not participate in the mating and the stalked cell is the likely recipient of the plasmid.

Chromosomal marker transfer mediated by RP1

Stanisich & Holloway (1971) showed that RP1 mediated chromosome transfer in isogenic strains of *Pseudomonas*. RP1 was further tested for its ability to mediate chromosomal marker transfer to *Caulobacter*. *Pseudomonas aeruginosa* PAO67 was mated with *C. crescentus* WS12, which is an auxotroph requiring methionine for growth. Plasmid transfer from *P. aeruginosa* to this *Caulobacter* strain was demonstrated by acquisition of resistance to kanamycin and tetracycline. Appropriate selective media were then used to test for transfer of the methionine marker. In the *Pseudomonas* × *Caulobacter* mating, transfer of the methionine marker, as determined by prototroph formation, was not observed. Since the transfer of a nutritional marker in a *Pseudomonas* × *Caulobacter* cross was unsuccessful, the same experiment was attempted in a *Caulobacter* × *Caulobacter* mating. First the RP1 plasmid was transferred from *P. aeruginosa* PAO67 to *C. vibrioides* CV6. Strain CV6 R⁺ was then mated with *C. crescentus* WS30, a derivative of strain WS12 resistant to streptomycin. *Caulobacter* strains WS30 and CV6 are morphologically identical: they both have a vibrioid cell form and similar mol % of guanine plus cytosine. However, strain CV6 requires riboflavin whereas strain WS30 does not (Poindexter, 1964). As a result of the mating, strain WS30 acquired resistance to kanamycin and tetracycline. To test for co-transfer of markers, the kanamycin-resistant colonies were tested in tetracycline. Approximately 50 % of the colonies resistant to kanamycin were also resistant to tetracycline suggesting a segregation of this antibiotic resistance marker. The formation of prototrophic recombinants in a *Caulobacter* CV6 R⁺ × WS30 cross would be evidence of transfer of a chromosomal marker, but no such evidence was found (Table 6) nor was microcolony formation observed which might suggest crossfeeding. When the mating mixture of *Caulobacter* strains CV6 R⁺ × WS30 was plated on kanamycin minimal selective medium lacking methionine, colony growth was observed. These colonies took 2 to 3 weeks to appear and did not grow to larger than 0.75 mm diam. Small colonies also appeared on minimal medium plus streptomycin, indicating transfer of the methionine marker alone; this transfer was about 30 % as efficient as transfer of the methionine and kanamycin markers together.

To test the stability of the methionine marker in *C. crescentus* WS30, colonies growing on medium lacking methionine were transferred to the same medium. Colonies failed to grow upon transfer, suggesting instability of this RP1-mediated chromosomal marker transfer.

Our data do not confirm mobilization of the *Caulobacter* chromosome but do suggest that a chromosomal marker is transferred. Random recombination between the *Caulobacter* chromosome and RP1 or chromosomal transfer could account for our observation.

The instability of the newly acquired methionine marker in the WS30 transcient could reflect transfer but failure to recombine, as is seen in abortive transduction.

Transfer of *Pseudomonas* plasmid RP1 to *Caulobacter* provides a means by which plasmid expression in this dimorphic bacterium can be studied. Plasmid expression and possibly chromosomal marker transfer may be used in the characterization of *Caulobacter*.

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