RP4-mediated Conjugation in *Acinetobacter calcoaceticus*

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SUMMARY

The P class R factor RP4 was transferred from *Escherichia coli* K12 to *Acinetobacter calcoaceticus*. RP4 conferred similar levels of antibiotic resistance in *A. calcoaceticus* to those in the *E. coli* K12 donor. Only slight instability of RP4 in *A. calcoaceticus* was detected. Transfer of RP4 between strains of *A. calcoaceticus* was by conjugation and was accompanied by transfer of chromosomal genes. Possible polarity of marker transfer was observed and linkage between a number of chromosomal markers was demonstrated.

INTRODUCTION

Interest in the Gram-negative coccobacilli now classified as *Acinetobacter calcoaceticus* (Bergey's *Manual of Determinative Bacteriology*, 1974) has recently been increasing. Apart from their possible pathogenic role (Henriksen, 1973), investigations have included studies on the tryptophan pathway (Twarog & Liggins, 1970), the isoleucine-valine pathway (Twarog, 1972) and pyrimidine biosynthesis (Øvrebo & Kleppe, 1973), whilst regulatory mechanisms have been investigated in the following pathways: L-mandelate (Livingstone & Fewson, 1972), benzyl alcohol (Livingstone *et al.*, 1972) and β -ketoadipate (Stanier & Ornston, 1973).

Genetical studies have so far been limited to the demonstration of transformation (Juni & Janik, 1969) and transduction (Herman & Juni, 1974). Both of these techniques suffer from the limitation that it is difficult to map large segments of the genome. The R factor experiments described in this paper were an attempt to determine the ability of *A. calcoaceticus* to transfer DNA by conjugation. RP4, an R factor of the compatibility class P, was chosen, as members of this class have been shown to be transferable between a large range of Gram-negative bacteria (Sykes & Richmond, 1970; Datta *et al.*, 1971; Roe, Jones & Lowbury, 1971; Datta & Hedges, 1972; Olsen & Shipley, 1973; Beringer, 1974). P class R factors have also been shown to mobilize the bacterial chromosome in *Pseudomonas aeruginosa* (Stanisich & Holloway, 1971). RP4 was originally isolated from *P. aeruginosa* and carries markers conferring resistance to ampicillin, kanamycin/neomycin and tetracycline (Holloway & Richmond, 1973).

METHODS

Bacterial strains used are listed in Table 1 together with their sources.

Media. All media were made with distilled water and were solidified when necessary with 1.5 % (w/v) Difco Bacto agar. NB was Oxoid nutrient broth. TSA was Oxoid tryptone soya agar. The minimal medium (MM) used was that of Twarog & Liggins (1970) and was supplemented with amino acids (50 μ g/ml) when required. Media were autoclaved for 15 min at 121 °C before use.

Antibiotics used to select for R factor transfer in *A. calcoaceticus* were freshly prepared

Table 1. *Bacterial strains*Wild-type strains of *Acinetobacter calcoaceticus*

Source	Strain number
Dr R. W. A. Park	154, 160, 166, 168
Dr E. M. Barnes	EBF34/373, EBF65/53, EBF65/65, EBF65/174, EBF65/185, EBF85/24
National Collection for Industrial Bacteria	8250, 9211, 9212, 9213, 9214, 9292, 9294, 9295, 9296, 9297, 9299, 9300, 9301, 9305
Auxotrophs of <i>A. calcoaceticus</i> EBF65/65	
C43	<i>met-1</i>
C44	<i>his-1</i>
C48	<i>ile-1 met-1</i>
C411	<i>arg-3 his-1</i>
C426	<i>trp-2 his-1</i>
C432	<i>thi-1 arg-3</i>
<i>Escherichia coli</i> K12	
Strain J5-3(RP4)	F ⁻ <i>pro met nal-r</i>
(source: Dr J. E. Beringer)	

solutions in distilled water and were added to media at the following final concentrations ($\mu\text{g/ml}$): kanamycin sulphate, 180; ampicillin sodium, 500; tetracycline HCl, 5. Ampicillin sodium was a gift from Beecham Research Laboratories, Brockham Park, Surrey. Other antibiotics were purchased from Sigma.

Mutagenesis. Auxotrophic mutants of *A. calcoaceticus* were induced either by ultraviolet irradiation at a wavelength of 254 nm or by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) using the procedure devised by Fargie & Holloway (1965) for *P. aeruginosa*. Multiply-marked mutants were produced by repeated treatment.

Plate mating technique. This method was used to detect R factor transfer to *A. calcoaceticus* and was based on the technique used by Hopwood *et al.* (1969) with *Streptomyces coelicolor*. Wild-type recipient cultures were grown as streaks on the surface of MM plates. These streaks were then replicated on to plates of TSA evenly spread with 0.1 ml of a dense suspension of the auxotrophic *Escherichia coli* K12 donor culture. After incubation at 28 °C for 24 h these plates were replicated on to plates of MM supplemented with either kanamycin, tetracycline or ampicillin to select for wild-type *A. calcoaceticus* strains which had received the R factor.

Millipore mating technique. This technique was used for all quantitative studies. Donor and recipient cultures were grown in 10 ml NB incubated for 18 h at 28 °C on a rotary shaker. Portions (5 ml) of each culture were mixed together and 3 ml of the resultant mixture filtered through a 0.45 μm Millipore filter. The filter was incubated for 24 h at 28 °C on the surface of a TSA plate and then vigorously whirlmixed for 1 min with 3 ml quarter-strength Ringer's solution in a wide-mouthed 50 ml conical flask to resuspend the cells. The resulting suspension was then plated on the appropriate selective media.

RESULTS

Host range for RP4 transfer

Olsen & Shipley (1973) reported that the P group R factor R1822 could be transferred to *A. calcoaceticus*. To determine whether RP4 could be transferred to a wide range of *A. calcoaceticus* strains, *E. coli* K12 strain J5-3(RP4) was crossed with the wild-type strains listed in Table 1 using the plate-mating technique described in Methods. All 24 of the strains

Table 2. *Transfer of RP4 on Millipore filters*

Crosses were performed as described in Methods. Where indicated (+) DNAase (1 ml) at 400 µg/ml in 0.2 M-MgSO₄ was added to 9 ml of mating mixture before filtering. Frequencies of transfer are expressed in terms of RP4⁺ recipients per recipient.

Cross		DNA ase	10 ⁵ × Frequency of transfer
Donor	Recipient		
J5-3(RP4)	C426	—	1.0
J5-3(RP4)	C426	+	1.3
C426(RP4)	C411	—	28
C426(RP4)	C411	+	27
C48(RP4)	C426	—	11
C48(RP4)	C426	+	14

tested had been randomly selected on the basis of their ability to grow on MM and all were found to acquire resistance to kanamycin, tetracycline and ampicillin. This demonstrates a general ability of strains of *A. calcoaceticus* to act as recipients of RP4 in crosses of this type. As a result of this work, strain EBF65/65 was selected for detailed quantitative study.

Transfer of RP4 on Millipore filters

RP4 was transferred from *E. coli* K12 strain J5-3 to *A. calcoaceticus* strain EBF65/65 on Millipore filters as described in Methods, both with and without the addition of DNAase (Sigma; beef pancreas). Similar crosses were also performed between auxotrophic mutants of EBF65/65. The results obtained (Table 2) show that in neither case was transfer inhibited significantly by the presence of DNAase. If transformation were occurring to any extent it would be expected that the presence of DNAase would at least cause a marked reduction in the frequency of transfer (Juni & Janik, 1969; Beringer, 1974).

All the antibiotic resistance markers carried by RP4 were expressed in *A. calcoaceticus* and levels of resistance were comparable to those found in the RP4-carrying *E. coli* donor.

Stability of RP4 in A. calcoaceticus

Six RP4-carrying strains of *A. calcoaceticus* were tested for the stability of the plasmid by plating for single colonies on antibiotic-free MM and then replicating on to plates of MM containing kanamycin, tetracycline or ampicillin. More than 1500 colonies of each strain were examined and in each case the number of colonies which appeared to have lost RP4 did not exceed 2 %. However, with longer periods of subculture on antibiotic-free MM the percentage of colonies which had lost RP4 gradually increased. No segregation of resistance determinants was observed: all three markers always appeared to be lost simultaneously.

Transfer of chromosomal genes in A. calcoaceticus

Crosses between auxotrophic mutants of EBF65/65 were performed to look for recombinant formation associated with R factor transfer. The results obtained (Table 3) indicated that recombinants were indeed formed, that the formation of such recombinants was not inhibited by the presence of DNAase, and that no recombinants were formed unless RP4 was present in one parent.

Initial observations on the frequencies of various classes of recombinants are presented in Table 4. Although the order of the various markers cannot be deduced from the different frequencies of recovery, the wide range of frequencies observed suggests that polarity of entry of markers could possibly be occurring.

Table 3. *Transfer of chromosomal genes*

Crosses were performed on Millipore filters as described in Methods. Where indicated DNAase (1 ml) at 400 µg/ml in 0.2 M-MgSO₄ was added to 9 ml of mating mixture before filtering. In each cross, selection was made for *ile⁺ met⁺* in the recipient and for either *his⁺* or *trp⁺* from the donor.

Cross		DNAase	No. of recombinants/10 ⁸ recipient cells	
Donor <i>ile-1 met-1</i>	Recipient <i>his-1 trp-2</i>		<i>his⁺</i>	<i>trp⁺</i>
c48(RP4)	c426	—	142	34
c48(RP4)	c426	+	148	36
c48	c426	—	0	0

Table 4. *Relative frequencies of different recombinant classes*

After mating on Millipore filters as described in Methods, the cells were plated on MM suitably supplemented to select for transfer of the indicated donor marker to the recipient. Selection was made against both auxotrophic markers contained in the donor.

Cross		Marker selected from donor	Average no. of recombinants/10 ⁸ recipient cells
Donor	Recipient		
c48(RP4)	c426	<i>his-1⁺</i>	146
<i>ile-1 met-1</i>	c426	<i>trp-2⁺</i>	44
	c411	<i>arg-3⁺</i>	11
	c432	<i>thi-1⁺</i>	6
c426(RP4)	c48	<i>met-1⁺</i>	4
<i>his-1 trp-2</i>	c48	<i>ile-1⁺</i>	2

Table 5. *Linkage between chromosomal markers*

Crosses were performed on Millipore filters as described in Methods. After mating, the cells were plated on MM suitably supplemented to select for transfer of the indicated donor marker to the recipient. Recombinants obtained were then replicated to test for co-transfer of the unselected second marker. At least 50 recombinants of each type were tested.

Donor	Recipient	Transferred donor markers		Percentage co-transfer
		Selected	Unselected	
c48(RP4) <i>ile-1 met-1</i>	c426	<i>his-1⁺</i>	<i>trp-2⁺</i>	19
	c426	<i>his-1⁺</i>	<i>met-1</i>	1
	c426	<i>trp-2⁺</i>	<i>his-1⁺</i>	60
	c426	<i>trp-2⁺</i>	<i>met-1</i>	9
	c411	<i>his-1⁺</i>	<i>arg-3⁺</i>	2
	c411	<i>arg-3⁺</i>	<i>his-1⁺</i>	28
	c411	<i>arg-3⁺</i>	<i>met-1</i>	33
	c411	<i>arg-3⁺</i>	<i>ile-1</i>	3
	c432	<i>thi-1⁺</i>	<i>arg-3⁺</i>	22
	c432	<i>thi-1⁺</i>	<i>met-1</i>	72
	c432	<i>thi-1⁺</i>	<i>ile-1</i>	8
c426(RP4) <i>his-1 trp-2</i>	c48	<i>met-1⁺</i>	<i>trp-2</i>	29
	c48	<i>met-1⁺</i>	<i>ile-1⁺</i>	14
	c48	<i>ile-1⁺</i>	<i>trp-2</i>	13
	c48	<i>ile-1⁺</i>	<i>met-1⁺</i>	53

Linkage between chromosomal markers

Table 5 presents the results of attempts to determine linkage between the various markers studied. Taking the percentage of recombinants between the pairs of markers listed as a measure of linkage, it can be concluded that all six markers are on one linkage group and the probable order is *his-trp-arg-thi-met-ile*.

DISCUSSION

The object of this investigation was to attempt to establish the existence of conjugal chromosomal gene transfer in *A. calcoaceticus*. The formation of auxotrophs from doubly auxotrophic parent strains (Table 5) could not be explained on the grounds of spontaneous back-mutation and was decisive evidence for genetic recombination. Transformation was excluded by the demonstration that the presence of DNAase in mating mixtures had no effect on the outcome of crosses. The fact that RP4 was essential for the formation of recombinants (Table 3) reinforces this belief, and coupled with the demonstration that a group of six linked genes on the chromosome were being transferred, makes it extremely unlikely that transduction was involved.

The possible polarity of entry of markers introduces the question of the relationship between RP4 and the donor chromosome. From the results obtained it would seem that some unstable association between the plasmid and the chromosome, with interaction in the histidine region, may be operative in the present system. This would mean that only a fraction of the donor population was capable of chromosome transfer (Adelberg & Burns, 1960), and would explain the much higher frequency of independent plasmid transfer. In the *P. aeruginosa* mating system a similar unstable association between sex factor and the chromosome has been proposed by a number of authors (Holloway & Fargie, 1960; Loutit, Pearce & Marinus, 1968; Stanisich & Holloway, 1969; Pemberton & Holloway, 1973). Certainly, results of mating experiments were not what would be expected if RP4 had many potentially equal sites of association with the *A. calcoaceticus* chromosome.

Interrupted mating experiments have not been attempted as the comparatively low recombination frequencies encountered would make it extremely difficult to obtain accurate times of entry. However, using the present system of chromosome transfer it should now be possible to proceed towards the construction of a genetic map for *A. calcoaceticus*.

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