Naturally Occurring Plasmids in *Acinetobacter calcoaceticus*: a P Class R Factor of Restricted Host Range

By EDWARD HINCHLIFFE AND ALAN VIVIAN

School of Biological Sciences, Thames Polytechnic, Wellington Street, London SE18 6PF

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A naturally occurring transmissible plasmid, designated pAV1, has been isolated in *Acinetobacter calcoaceticus*. It specifies resistance to sulphonamides and is capable of mobilizing two non-transmissible resistance determinants for tetracycline and neomycin, respectively, within strains of *A. calcoaceticus*. It is incompatible with the P class R factors RP4 and R751 in *A. calcoaceticus*. On this basis we conclude that pAV1 is a member of the P incompatibility group. However, unlike most other P group R factors, pAV1 is not transmissible to strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* or *Proteus mirabilis*.

INTRODUCTION

Towner & Vivian (1976*a, b*) have used the plasmid RP4 to mobilize the chromosome in a strain of *Acinetobacter calcoaceticus*. Using this system of gene transfer and analysis of coinherance frequencies for markers, a circular linkage map was established for the strain EBF65/65 (Towner, 1978). RP4 mobilizes the chromosome from one of two alternative origins, resulting in a polarized transfer of markers from the donor to the recipient strain. Inheritance of markers distal to both donor origins is rather low, making it difficult to resolve the order of markers in this region of the map (Towner, 1978).

We have continued the search for a more efficient sex factor than RP4 among strains of *A. calcoaceticus*. In this paper we report the discovery and genetic characterization of a plasmid, designated pAV1, which was obtained from strain JC17, a hospital isolate of *A. calcoaceticus*. pAV1 apparently has a very restricted host range and for this reason we have found it necessary to perform incompatibility experiments in strain EBF65/65, using P group plasmids, as this is the only group so far shown to transfer to EBF65/65 (Towner & Vivian, 1977).

METHODS

Acinetobacter strains and plasmids. These are listed in Table 1 together with their sources. *Escherichia coli* J53.2 (Bachmann, 1972) and *Pseudomonas aeruginosa* OT47 (Loutit *et al.*, 1968) were used for host range experiments, as well as two strains of *Proteus mirabilis* and two strains of *Klebsiella* sp. supplied by Dr S. Poston, Guys Hospital Medical School, London.

Media. Minimal medium (MM), Tryptone Soya Agar (TSA) (Towner & Vivian, 1976*a*) and basal salts medium (BSM) (Towner, 1978) were used. Minimal medium used for strains other than *A. calcoaceticus* was that of Vogel & Bonner (1956), supplemented with glucose at a final concentration of 0.5% (w/v). Oxoid nutrient agar (NA) was used for some host range experiments, and Oxoid Diagnostic sensitivity test agar was used for antibiotic sensitivity tests.

Determinations of antibiotic sensitivity. These were performed using Oxoid Multodiscs.

Antibiotics. These were obtained from Sigma (except where indicated) and were added to media as freshly prepared solutions at the following final concentrations: sulphadiazine, 500 µg ml⁻¹; rifampicin, 100 µg ml⁻¹; tetracycline hydrochloride, 5 µg ml⁻¹; neomycin sulphate, 50 µg ml⁻¹; trimethoprim lactate (Wellcome), 500 µg ml⁻¹.

Table 1. *Strains of Acinetobacter calcoaceticus and plasmids*

Strains	Relevant characteristics*	Source
<i>Bacteria</i>		
EBF65/65	Km Ap	Towner & Vivian (1976a)
EBF65/174		E. M. Barnes, Norwich
8250, 9299		National Collection of Industrial Bacteria
JC17	Tc Nm Ap Km Su (pAV1)	J. N. Coetzee, Pretoria, South Africa
C48	<i>met-1 ile-1</i>	} Auxotrophs of EBF65/65
C426	<i>trp-2 his-1</i>	
C4121	<i>trp-2 his-1 rif-1</i>	
C4131	<i>phe-1 thi-2</i> Tp (R751)	
C4145	<i>trp-2 his-1</i> Tc Nm Su (pAV1)	
C4151	<i>trp-2 his-1</i> Su (pAV1)	
C4152	<i>met-1 ile-1</i> Su (pAV1)	
C4156	<i>trp-2 his-1</i> Tc Km Ap (RP4)	
<i>Plasmids</i>		
pAV1	Su	This paper
RP4	Tc Km Ap	Datta <i>et al.</i> (1971)
R751	Tp	Jobanputra & Datta (1974)

* Abbreviations (resistance to): Ap, ampicillin; Km, kanamycin; Nm, neomycin; Su, sulphonamides; Tc, tetracycline; Tp, trimethoprim.

Millipore mating technique. The method of Towner & Vivian (1976b) was used for all quantitative crosses.

Replica plate mating. This was done essentially as described by Hopwood *et al.* (1969). Donor cultures were plated for single colonies on TSA; these single colonies were then replicated to plates of MM plus the appropriate antibiotic, and to plates of TSA evenly spread with 0.1 ml of a recipient culture. After incubation at 28 °C for 24 h mating plates were replicated to selective media.

Selection for R factor transfer. Selection was made by Millipore mating between JC17 and C4121 and plating on BSM supplemented with D(–)-mandelic acid (Sigma) at a final concentration of 5 mM. This selection was necessary because of the relatively high mutation rate to rifampicin resistance and D(–)-mandelate utilization in JC17. We were unable to select directly for pAV1 transfer by this method, since no transconjugants were obtained that were resistant to both rifampicin and sulphonamides.

Stability of plasmids. This was determined as described by Towner & Vivian (1976b).

Incompatibility studies with pAV1. These were done in *A. calcoaceticus* EBF65/65 with the plasmids RP4 and R751, using the Millipore mating technique. Transconjugants were streaked on TSA and replicated to check for the inheritance of resistance markers. The stability of clones carrying resistance markers of two different plasmids was determined as described by Datta & Hedges (1971).

Host range of pAV1. This was determined by the Millipore mating technique. Filters were additionally incubated on NA at 37 °C in all crosses involving recipient strains other than *A. calcoaceticus*.

RESULTS

Transfer of resistance determinants

Strain JC17 is one of a collection of *Acinetobacter* cultures originally isolated from patients in a hospital in Pretoria, South Africa. Many of these strains show multiple drug resistance; JC17 is resistant to tetracycline, sulphonamides, neomycin, kanamycin and ampicillin, and is unable to utilize D(–)-mandelic acid as a carbon source. Strain EBF65/65 and its derivatives are able to utilize D(–)-mandelic acid as a carbon source and are also resistant to kanamycin and ampicillin. For the reasons indicated in Methods, it was only possible to select for transfer of tetracycline resistance into strain EBF65/65. Tetracycline-resistant transconjugants were obtained in strain C4121 at a frequency of 4.0×10^{-6} per recipient cell. Examination of these transconjugants showed that most were also resistant to neomycin, and a small proportion (11 in 400) were also resistant to sulphonamides.

Subsequent replica plate mating of a sample of 49 transconjugants with the recipient strain C48 indicated that only 11, all of which had acquired sulphonamide resistance, were

Table 2. Frequency of R factor transfer in auxotrophic strains of *A. calcoaceticus* EBF65/65

Donor	Recipient	Resistance marker* selected from donor	Resistance marker* unselected from donor	Frequency of transfer (Su R ⁺ transconjugants per recipient)	Characteristics of transconjugants*‡
C4151	C48	Su	—	2.0×10^{-1}	305 (1500) Tc ^R Nm ^R
C4152	C426	Su	—	2.0×10^{-1}	
C4145	C48	Su	—	2.0×10^{-1}	
		Tc	Su	6.4×10^{-2}	543 (547) Su ^R
		Tc	—	$6.4 \times 10^{-2} \dagger$	
		Nm	—	$4.8 \times 10^{-2} \dagger$	359 (366) Su ^R

* Nm, neomycin; Tc, tetracycline; Su, sulphonamides.

† Frequency of transfer expressed as Tc and Nm R⁺ transconjugants, respectively, per recipient.

‡ Numbers in parentheses indicate the total number of colonies tested.

Table 3. Incompatibility properties of plasmids in EBF65/65

Donor	Recipient	Selected plasmid resistance*	Frequency of R factor transfer†	Characteristics of transconjugants*‡
C4152(pAV1)	C4156(RP4)	Su	1.3×10^{-2}	137 (137) Su ^R Tc ^S (RP4 eliminated)
C4152(pAV1)	C4131(R751)	Su	1.4×10^{-2}	80 (80) Su ^R Tp ^S (R751 eliminated)
C4156(RP4)	C4152(pAV1)	Tc	7.3×10^{-2}	522 (524) Tc ^R Su ^R (pAV1 retained)
C4131(R751)	C4152(pAV1)	Tp	8.0×10^{-3}	165 (166) Tp ^R Su ^R (pAV1 retained)
C4156(RP4)	C4131(R751)	Tc	5.6×10^{-4}	246 (246) Tc ^R Tp ^S (R751 eliminated)
C4131(R751)	C4156(RP4)	Tp	3.8×10^{-3}	208 (208) Tp ^R Tc ^S (RP4 eliminated)
C4156(RP4)	C48	Tc	8.7×10^{-4}	
C4131(R751)	C48	Tp	1.0×10^{-2}	

* Su, sulphonamides; Tc, tetracycline; Tp, trimethoprim.

† R⁺ transconjugants per recipient.

‡ Numbers in parentheses indicate the total number of colonies tested.

able to mediate further transfer of either tetracycline, neomycin or sulphonamide resistance. Millipore matings between C4145 and C48 resulted in the segregation of the sulphonamide resistance determinant from the tetracycline and neomycin resistance determinants amongst the transconjugants (Table 2). Segregation of neomycin and tetracycline determinants from each other was not observed in crosses of this type. Consequently it was concluded that the sulphonamide resistance determinant is associated with a self-transmissible plasmid, designated pAV1. Two other resistance determinants are associated with resistance to tetracycline and neomycin, respectively; neither is self-transmissible in EBF65/65. It is not clear at this stage whether these two determinants constitute a single replicon (E. Hinchliffe, A. Vivian & R. W. Hedges, unpublished results).

Millipore matings with transconjugants carrying pAV1 showed that it was transferred at much higher frequencies within derivatives of EBF65/65, whether selection was made directly for sulphonamide resistance or indirectly for tetracycline resistance (Table 2).

Table 4. *Stability of R factors in pairs in A. calcoaceticus* EBF65/65

Strain	Resistance markers*	No. of colonies tested	No. of colonies that did not grow on plates containing*:		
			Su	Tp	Tc
C4152(pAV1)RP4 ⁺	Su, Tc	1587	3	—	1564
C4152(pAV1)R751 ⁺	Su, Tp	1043	3	1043	—

* Su, sulphonamides; Tc, tetracycline; Tp, trimethoprim.

Properties of pAV1

Stability in strain EBF65/65. pAV1 is very stable in strain EBF65/65. A sample of 1247 colonies cultured on antibiotic-free media retained sulphonamide resistance and sex-factor activity in 1243 colonies. Sex-factor activity was always associated with sulphonamide resistance, as indicated by mobilization of the non-transmissible resistance determinants.

Host range. pAV1 was unable to transfer itself at any detectable frequency ($< 1 \times 10^{-8}$ R⁺ transconjugants per recipient) to other species of Gram-negative bacteria tested. These included the strains of *Klebsiella*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *E. coli* listed in Methods. pAV1 did not transfer to any of the other wild-type strains of *A. calcoaceticus* listed in Table 1.

Incompatibility studies. Crosses were made between appropriately marked strains carrying one of the plasmids pAV1, RP4 or R751 so that all pairwise combinations of plasmids could be tested (Table 3). The mating mixtures were plated on media selecting for reciprocal transfer of resistance markers between parent strains, and the phenotypes of the resulting transconjugants were tested by replica plating to appropriate drug-supplemented media. Transfer between pAV1⁺ recipients and either RP4⁺ or R751⁺ donor strains occurred at a relatively high frequency, without apparent evidence of entry exclusion. However, the reciprocal transfer of pAV1 was slightly reduced (approximately 10-fold) by RP4 or R751 in the recipient. RP4 in the recipient did not exhibit strong entry exclusion of R751, unlike the case in *E. coli* K12 where there is an approximately 1000-fold reduction in the transfer of R751 (Hedges & Jacob, 1974). Further, whenever selection was made for the transfer of pAV1 into an R⁺ strain, there was a concomitant displacement of the resident plasmid; this was also true for crosses between R751 and RP4 and has been described for crosses involving R906 (Hedges *et al.*, 1974) and for RP4 and R751 (Hedges & Jacob, 1974). However, when selection was made for the transfer of a P class R factor into a pAV1⁺ strain, only a very small proportion of transconjugants showed sulphonamide sensitivity. Transconjugant colonies selected for tetracycline resistance from the cross involving RP4 and pAV1 were found to be of two different sizes. Large colonies carried resistance to tetracycline alone, whereas smaller colonies invariably were resistant to both tetracycline and sulphonamide, and it was therefore concluded that they carried both RP4 and pAV1.

The stability of pairs of plasmids amongst transconjugants was tested by subculturing and replicating to check for the continued presence of both R factors. The results suggested that co-existence of pAV1 with either RP4 or R751 did not persist for more than a few generations, and the plasmid retained was always pAV1 (Tables 3 and 4). A similar preferential retention of plasmid R772 over R751 in *E. coli* K12 has recently been described by Coetzee *et al.* (1979).

DISCUSSION

The evidence presented in this paper indicates that a self-transmissible plasmid specifying resistance to sulphonamides has been transferred from a hospital isolate of *A. calcoaceticus* into the genetically mapped strain EBF65/65. This plasmid, designated pAV1, is unusual in

that it appears to be a member of the P incompatibility group of *E. coli*, but is restricted to certain strains of *A. calcoaceticus* in its host range.

There is no significantly large reduction in reciprocal resistance transfer between the P class plasmids and pAV1, unlike the situation described for the I and N group of plasmids (Datta & Hedges, 1971), and the P group R factors RP4, R751 and R906 (Hedges & Jacob, 1974; Hedges *et al.*, 1974). The frequencies of reciprocal resistance transfer involving pAV1 (Table 3) are 10- to 100-fold higher than those involving RP4 and R751 alone. This enhanced frequency of transfer of RP4 and R751 may be due to less fragile conjugal unions in which pAV1 is involved, or alternatively pAV1-mediated mobilization following superinfection by this more efficient R factor.

The displacement of RP4 and R751 by pAV1 under non-selective conditions (Table 4) is good evidence that pAV1 is a member of the P incompatibility group of plasmids (Novick *et al.*, 1976). Further evidence in support of this thesis is the formation of large and small transconjugant colonies in crosses involving RP4 and pAV1, when selected as tetracycline-resistant. We attribute this phenomenon to a small proportion of cells carrying RP4 in a colony which is predominantly pAV1⁺. Consequently these few cells confer residual tetracycline resistance on their parent colony. Datta & Hedges (1971) showed that the plasmid most frequently retained in stability studies of this kind is the one whose resistance marker is selected in the cross. In contrast, pAV1 may be retained in preference to RP4 and R751, because it has a much higher affinity for the *A. calcoaceticus* host. One might expect this result if pAV1 is a true naturally occurring *Acinetobacter* sex factor.

Unlike other members of the P incompatibility group of plasmids, pAV1 has a limited host range (Datta *et al.*, 1971; Datta & Hedges, 1972; Olsen & Shipley, 1973), although Terakado & Mitsuhashi (1974) have reported a narrowed host range for the P class plasmid Rte16 alias R906 (Hedges *et al.*, 1974), isolated from *Bordetella bronchiseptica*. The inability of pAV1 to transfer to other Gram-negative bacteria may be limited by the intrinsic incompatibility of plasmid-specified donor structures and recipient sites. Alternatively stable pAV1⁺ recipient formation may be limited by the inability of pAV1 to replicate in a foreign host. Whichever alternative is true, our findings may be indicative of some degree of specialization amongst members of the P-I incompatibility group.

It is obviously of interest to investigate the relationship between the plasmid pAV1, described here, and the non-transmissible tetracycline and neomycin resistance determinants, since this might shed some light on the importance of relatively non-pathogenic bacteria in the spread of antibiotic resistance.

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