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Characterization of an antibiotic resistance plasmid pAV5 and its constituent replicons in *Acinetobacter calcoaceticus*

BY MARK DIVERS, EDWARD HINCHLIFFE* AND ALAN VIVIAN

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

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SUMMARY

A non-transmissible plasmid, pAV5, was isolated from a hospital strain of *Acinetobacter calcoaceticus*, JC17. pAV5 confers resistance to the antibiotics tetracycline and neomycin in the genetically characterized strain of *A. calcoaceticus* EBF 65/65. Transfer of pAV5 can be mediated by the sex factors pAV1 and R751; transfer is occasionally associated with the segregation of the resistance determinants amongst the transconjugants. Phenotypic dissociation of pAV5 corresponds with the formation of two independent plasmids designated pAV51 and pAV52 mediating resistance to neomycin and tetracycline respectively.

1. INTRODUCTION

Acinetobacter calcoaceticus is a Gram-negative, oxidase-negative, aerobic coccobacillus which occurs widely in nature (Juni, 1978). It has recently assumed some importance as an opportunistic pathogen in hospital-acquired infections, due mainly to modern clinical practices involving immunosuppressive therapy and instrumentation of patients (Ramphal & Kluge, 1979). Many hospital isolates exhibit multiple resistance to antibiotics, and the present study is aimed at understanding the genetic basis of this resistance (Vivian, Hinchliffe & Fewson, 1981).

Hinchliffe, Nugent & Vivian (1980) reported the occurrence of a plasmid of approximately 30 megadaltons (MDal) in a hospital isolate (JC17) of *Acinetobacter calcoaceticus*. This plasmid, for which we propose the designation pAV5, specified resistance to tetracycline and kanamycin/neomycin in JC17, but owing to the presence of an intrinsic high-level resistance to kanamycin in EBF 65/65, only resistance to tetracycline and neomycin could be demonstrated on transfer to the latter strain (Hinchliffe & Vivian, 1980*a*). The resistances specified by pAV5 were non-transmissible in EBF 65/65 strains, but could be mobilized by the transmissible sulphonamide resistance plasmid, pAV1 (Hinchliffe & Vivian, 1980*a*).

In this paper we describe the behaviour of pAV5 in two strains of *A. calcoaceticus* and the conditions under which it can be observed to dissociate, resulting in the

* Present address: Research and Development Laboratory, Bass Brewing Ltd, 137 High Street, Burton-on-Trent DE14 1JZ.

formation of two independent replicons, pAV51 which mediates resistance to neomycin and pAV52 which mediates resistance to tetracycline.

2. MATERIALS AND METHODS

Acinetobacter strains and plasmids are listed in Table 1 with their derivation.

Media were as described by Hinchliffe & Vivian (1980*a*). All cultures were incubated at 28 °C. TSA is trypticase soy agar.

Filter matings were as described by Towner & Vivian (1976*b*).

Antibiotics and other chemicals were obtained from Sigma except where indicated, and were added to media as freshly prepared solutions at the following final concentrations (μg ml^{-1}): neomycin sulphate, 50; sulphadiazine 500; tetracycline hydrochloride, 5; trimethoprim lactate (Burroughs Wellcome), 500.

Plasmid stability determinations were performed as described previously (Towner & Vivian, 1976*b*).

Agarose gel electrophoresis. A modification of the method of Eckhardt (1978), devised at the John Innes Institute, was used as follows. Bacteria harvested from 1·5 ml overnight nutrient broth cultures were resuspended in 30 μl of a freshly prepared lysis solution (consisting of 25% w/v sucrose, 50 mM Tris-HCl pH 8·0, 1 mg ml^{-1} lysozyme and 1 unit ml^{-1} preheated ribonuclease A) and maintained on ice for 1 h. The cells were then loaded on a horizontal agarose gel (0·7% w/v) in which had been cast a 2 mm wide plug of SDS-agarose (1% (w/v) SDS, 0·4% (w/v) agarose) running immediately behind the wells. Gels were run slowly at 20 V for 1 h and then at 60 V for 19 h followed by staining with ethidium bromide (1 μg ml^{-1}). After destaining, gels were visualized on a transilluminator Model C63 (UV Products Inc., California, U.S.A.).

3. RESULTS

(i) *pAV1- and R751-mediated transfer of pAV5*

Strain EBF 65/65 possesses a plasmid pAV2, which influences the frequency of plasmid transfer in strains harbouring it, by means of a restriction/modification system (Hinchliffe *et al.* 1980; Hinchliffe & Vivian, 1980*c*). Filter matings were set up between strains harbouring pAV5 and one of the two transmissible plasmids, pAV1 or R751: the donor strains differed in that some also possessed pAV2, while others did not. The results (Table 2) indicate that pAV5 inheritance is reduced in pAV2 recipients when the donor strain lacks pAV2, suggesting that some restriction of incoming unmodified pAV5 has occurred. These results also indicate that R751 is also able to mediate transfer of pAV5, at frequencies comparable to those obtained with pAV1.

(ii) *Segregation of tetracycline and neomycin resistance among pAV5 transconjugants*

Transconjugants, selected as either tetracycline- or neomycin-resistant from both pAV1- and R751-mediated transfer of pAV5, were replica plated to determine their inheritance of unselected resistances. The results (Table 3) show that when the pAV1$^+$ pAV2$^+$ donor strain C4145 was crossed with the pAV2$^+$ recipient C484,

Table 1. *Strains of* Acinetobacter calcoaceticus *and plasmids*

Strain	Relevant characteristics*	Source
JC17	pAV1, pAV5	Hinchliffe & Vivian, 1980*a*
EBF 65/65	Wild-type pAV2: all derivative strains prefixed C4	Towner & Vivian, 1976*a*
C48	*ile-1*, *met-1* pAV2	Hinchliffe & Vivian, 1980*a*
C484	*phe-1*, *thi-2* pAV2	Hinchliffe & Vivian, 1980*b*
C4121	*his-1*, *trp-2*, *rif-1*, pAV2	Hinchliffe & Vivian, 1980*a*
C4131	*phe-1*, *thi-2* pAV2, R751	Hinchliffe & Vivian, 1980*a*
C4141	*his-1*, *trp-2*, *rif-1* pAV1, pAV5	Hinchliffe & Vivian, 1980*b*
C4143	*his-1*, *trp-2*, *rif-1* pAV5	Spontaneous variant from C4141
C4144	*his-1*, *trp-2*, *rif-1* pAV52	Spontaneous variant from C4141
C4145	*his-1*, *trp-2*, *rif-1* pAV1, pAV2, pAV5	Hinchliffe & Vivian, 1980*b*
C4148	*his-1*, *trp-2*, *rif-1* pAV2, pAV52	Spontaneous variant from C4145
C4153	*ile-1*, *met-1* pAV1	Hinchliffe & Vivian, 1980*b*
C4161	*phe-1*, *thi-2*	Hinchliffe *et al.*, 1980
C4169	*his-1*, *trp-2*	Hinchliffe *et al.*, 1980
C4312	*phe-1*, *thi-2* pAV2, pAV51	C4141 × C484
C4313	*phe-1*, *thi-2* pAV1, pAV2, pAV51	C4153 × C4312
C4314	*his-1*, *trp-2*, *rif-1* pAV5, R751	C4131 × C4143
C4316	*his-1*, *trp-2*, *rif-1* pAV1, pAV2, pAV52	C4148 × C4153
C4317	*his-1*, *trp-2*, *rif-1* pAV1, pAV51, pAV52	C4313 × C4144
BD413	Wild type: all derivate strains prefixed C9	Professor E. Juni, University of Michigan, Ann Arbor, Mi 48109, USA
C91	Ura$^-$, Rif-r, Str-r	Dr J. T. Singer, University of Georgia, Athens, Ga 30602, USA
C915	Ura$^-$, Rif-r, Str-r, pAV52	C91 × JC17
C916	Ura$^-$, Rif-r, Str-r, pAV51	C91 × JC17
C917	Ura$^-$, Rif-r, Str-r, PAV5	C91 × JC17
Plasmids		
pAV1	Sul-r self-transmissible	Hinchliffe & Vivian, 1980*a*
pAV2	Specifies a restriction/ modification system	Hinchliffe *et al.* 1980
pAV5	Kan-r/Neo-r, Tet-r non-transmissible	This paper
pAV51	Kan-r/Neo-r non-transmissible	This paper
pAV52	Tet-r non-transmissible	This paper
R751	Tmp-r non-transmissible	Jobanputra & Datta, 1974

* Abbreviations: Kan, kanamycin; Neo, neomycin; Sul, sulphonamide; Tet, tetracycline; Tmp, trimethoprin.

no segregation of neomycin and tetracycline was observed amongst the transconjugants: these observations held true for transconjugants selected as resistant to each antibiotic in turn. In contrast, when pAV1$^+$ pAV2$^-$ or R751$^+$ pAV2$^-$ donor strains were similarly crossed, segregation of neomycin and tetracycline resistance was observed for transconjugants selected as neomycin-resistant.

Table 2. *Mobilization of pAV5 by pAV1 and R751*

(Filter matings were as described in Materials and Methods. All recipient strains possessed pAV2.)

Donor	Recipient	Selected resistance	Frequency of transconjugants per recipient ($\times 10^{-2}$)
C4141 (pAV1, pAV5)	C48	Tet	0·45
		Neo	1·6
	C484	Tet	0·06
		Neo	0·18
C4145 (pAV1, pAV2, pAV5)	C48	Tet	9·0
		Neo	6·4
	C484	Tet	8·6
		Neo	9·9
C4314 (R751, pAV5)	C484	Tet	0·07
		Neo	0·07

In order to determine whether a similar type of segregation phenomenon occurred in matings with a pAV2$^-$ recipient, crosses were made with strain C4161; in these crosses no segregation of the neomycin and tetracycline resistance determinants was ever detected (Table 3).

(iii) *The molecular nature of pAV5 and its derivative replicons*

The observation that the resistance determinants of pAV5 underwent segregation following conjugal transfer from pAV2$^-$ to pAV2$^+$ strains of EBF 65/65 posed some interesting questions regarding the molecular nature of the plasmid. Previous studies had indicated the presence of two plasmids in JC17: an 85 Mdal plasmid subsequently shown to correspond to pAV1 and a 30 Mdal plasmid, presumed to correspond to pAV5. However, because of the presence of several cryptic plasmids in strain EBF 65/65 it was difficult to interpret the physical evidence from agarose gels of transconjugants harbouring pAV5 (Hinchliffe *et al.* 1980). Consequently, it was decided to investigate the physical nature of pAV5 in a plasmid-free host of *A. calcoaceticus*. The strain chosen (designated C91) was derived from the microcapsule strain BD413 described by Juni & Janik (1969).

A filter mating between JC17 and strain C91 indicated that segregation of the two resistance determinants of pAV5 could also be obtained under these conditions of transfer (Table 4). From this cross transconjugants that had inherited resistance to tetracycline and neomycin (C917) and to each separately (C916 Neo^R, C915 Tet^R, C916 has also inherited pAV1) were isolated. These strains were examined for plasmid DNA by agarose gel electrophoresis. Strains JC17 and C917 had a

Table 3. *Segregation of resistance determinants amongst transconjugants derived from pAV1- and R751-mediated mobilization of pAV5*

(Filter matings were performed as in Materials and Methods. For details of characterization of transconjugants see text.)

Donor	Recipient	Selection	No. of transconjugants tested	No. of transconjugants resistant to the following combinations of antibiotics: Tet	Neo	Sul	Tet/Neo	Tet/Sul	Neo/Sul	Tet/Neo/Sul
C4141	C484	Tet	257	0	—	—	253	0	—	4
(pAV1, pAV5)	(pAV2)	Neo	172	—	26	—	146	—	0	0
		Sul	100	—	—	77	—	0	0	13
C4141	C4161	Tet	104	0	—	—	3	2	—	99
(pAV1, pAV5)		Neo	953	—	0	—	0	—	0	953
		Sul	101	—	—	83	—	0	0	18
C4145	C484	Tet	632	0	—	—	19	0	—	613
(pAV1, pAV2, pAV5)	(pAV2)	Neo	553	—	0	—	15	—	0	538
		Sul	99	—	—	79	—	0	0	20
C4145	C4161	Tet	159	0	—	—	2	0	—	157
(pAV1, pAV2, pAV5)		Neo	134	—	0	—	3	—	0	131
		Sul	98	—	—	81	—	0	0	17
				Tet	Neo	Tmp	Tet/Neo	Tet/Tmp	Neo/Tmp	Tet/Neo/Tmp
C4314	C484	Tet	708	0	—	—	708	0	—	0
(pAV5, R751)	(pAV2)	Neo	438	—	8	—	430	—	0	0
		Tmp	89	—	—	58	—	0	0	31
C4314	C4161	Tet	110	0	—	—	110	0	—	0
(pAV5, R751)		Neo	103	—	0	—	103	—	0	0
		Tmp	164	—	—	104	—	0	0	60

plasmid band which was about 23 kilobase pairs (kb) long, corresponding to pAV5. Strain C916 had a plasmid (designated pAV51) of about 12·5 kb carrying the neomycin-resistance determinant and strain C915 had a plasmid (pAV52) of about 17 kb carrying the tetracycline-resistance determinant. The approximate molecular sizes have been determined by restriction enzyme analysis of purified plasmid DNA (M. Divers and A. Vivian, unpublished results).

Table 4. *Transfer of antibiotic resistance from* A. calcoaceticus *strain JC17 (pAV1, pAV5) to strain C91*

(Filter mating as described in Materials and Methods.)

Selected resistance in strain C91	Frequency of transconjugants per recipient ($\times 10^{-3}$)	Inheritance of unselected resistances				Total colonies tested
		Tet	Tet/Neo	Tet/Sul	Tet/Neo/Sul	
Tet	1·04	2	29	1	136	168
		Neo	Neo/Tet	Neo/Sul	Neo/Tet/Sul	
Neo	0·79	0	0	1	132	133
		Sul	Tet/Sul	Neo/Sul	Tet/Neo/Sul	
Sul	2·08	24	0	2	11	37

(iv) *Properties of pAV5 and its antibiotic-resistant segregants*

The physical and genetic data show that pAV5 is capable of dissociation upon pAV1- and R751-mediated conjugational transfer into one of two replicons, mediating resistance to tetracycline and neomycin respectively. It is clearly of interest to know whether pAV5 itself is a co-integrate plasmid formed by recombination between two independent replicons, or whether it is a product of an association between two resistance determinants and a single unit of replication. In an attempt to understand more about pAV5 experiments were performed to investigate the genetic properties of the component plasmids pAV51 and pAV52.

Strains C4141 and C4145 (Table 1) were subjected to a stability test soon after their isolation from the initial cross between JC17 and C4121. The results indicated a high degree of instability of neomycin resistance in both strains (5 sensitive in 141 tested and 7 sensitive in 130 tested for C4141 and C4145 respectively); however, tetracycline resistance was 100% stable. When the stability of pAV5 in C4141 and C4145 was tested after prolonged subculture on TSA slopes supplemented with Tet, both resistance determinants were 100% stable (3570 and 2265 colonies tested respectively). Similarly, when the stabilities of tetracycline resistance in strain C4148 and neomycin resistance in C4312 were determined no antibiotic-sensitive segregant was obtained (2146 and 2452 colonies tested, respectively), indicating that each replicon was very stable.

(v) *Independent mobilization of pAV51 and pAV52*

Strains C4316 (Tet^R) and C4313 (Neo^R) were filter mated with C48 and C4169 respectively, and selection was made for the transfer of tetracycline and neomycin resistance where appropriate. Transfer of both pAV51 and pAV52 mediated by

pAV1 gave $7{\cdot}6 \times 10^{-3}$ and $2{\cdot}7 \times 10^{-2}$ R^+ transconjugants per recipient respectively; thus pAV1-mediated mobilization is not restricted to either pAV51 or pAV52, nor to pAV5 itself.

(vi) *Incompatibility*

To test whether pAV51 and pAV52 were incompatible with one another, strain C4313 (Neo^R) was filter mated with C4144 (Tet^R). A sample of 40 neomycin-resistant transconjugants was replica plated to test for resistance to sulphonamides and tetracycline; all 40 neomycin-resistant strains tested retained tetracycline resistance, however only 36 had received pAV1, as determined by their resistance to sulphonamides. (One of these was isolated as strain C4317). When the stability of tetracycline and neomycin resistance was tested under non-selective conditions in strain C4317, none of 250 colonies tested showed any loss of resistance. To determine whether the resistance determinants had re-associated to form a single plasmid, C4317 was filter mated with C484 and the transconjugants were replica plated to test for inheritance of the different resistance determinants. For the resistances specified by pAV51 and pAV52, transconjugants generally inherited only the resistance that was selected (132/133 Tet^R and 188/191 Neo^R); the remaining 4 transconjugants inherited both resistances. Similarly, when transconjugants were selected on sulphonamide 1/35 also inherited Neo^R; 10/35 also inherited Tet^R and the remaining 24 inherited only pAV1. Thus no transconjugant was obtained which was resistant to all three antibiotics, no matter which selection was made. This suggests that pAV51 and pAV52 had not re-associated in strain C4317, and exist as autonomously replicating compatible plasmids.

4. DISCUSSION

The evidence presented in this paper is consistent with the notion that pAV5 is a single plasmid which mediates resistance to tetracycline and neomycin, but which can undergo dissociation upon sex-factor-mediated mobilization. The estimated size of pAV5 based on restriction enzyme analysis (data not shown) is 23 kb. This is considerably less than the previous size estimate, which was based on single-colony lysates of strain JC17: it was consistently difficult to visualize the plasmid content of this strain by this technique, and this accounts for the discrepancy. The genetic dissociation of pAV5 corresponds to a physical dissociation into two replicons: pAV51 a plasmid of approximately 12·5 kb and pAV52 a plasmid of 17 kb. The fact that the sum of the molecular sizes for pAV51 and pAV52 does not correspond to that of pAV5 implies that plasmids pAV51 and pAV52 either share DNA homology or that sequences have been acquired from elsewhere during the process of dissociation.

It is not clear why the $pAV1^+$, $pAV5^+$ transconjugants in EBF 65/65 (strains C4141, C4145) were initially unstable for neomycin resistance (Table 1) but subsequently became stable. It is possible that a stable variant was inadvertently selected in each case.

The genetic data suggest that pAV51 and pAV52 are compatible plasmids, capable of autonomous replication within the same cell, since no segregation of the

resistance determinants was observed in strain C4317. This apparent stability could not be explained in terms of re-association between the replicons, since each was inherited independently upon pAV1-mediated mobilization, unlike the pAV1-mediated mobilization of pAV5, in which the tetracycline- and neomycin-resistance determinants co-segregate. This would imply that pAV5 is a co-integrate plasmid formed by recombination between two independent replicons, rather than a product of an association between two resistance determinants and a single unit of replication; although one cannot rule out the possibility that either pAV51 or pAV52 has acquired additional DNA for replication. Interestingly both pAV51 and pAV52 retain the ability to be mobilized by pAV1, The frequencies of transfer being similar to those reported for pAV5 itself. This may be indicative of a similarity in the mechanism of transfer between all three plasmids, and could be due to common genetic information following a duplication during the process of dissociation. Alternatively the similarity in transfer frequencies may be coincidental, being an intrinsic property of pAV51 and pAV52. Reversible *recA*-independent recombination has been shown to occur between plasmids Col EI or Col K and a naturally occurring miniplasmid (pLG 500), following sex-factor-mediated mobilization, analogous to that described here for the dissociation of pAV5 (Broome-Smith, 1980). Whether this is a feasible mechanism for the formation pAV5 and its subsequent dissociation remains to be determined.

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