Naturally Occurring Plasmids in *Acinetobacter calcoaceticus*: pAV2, a Plasmid which Influences the Fertility of the Sex Factor pAV1

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Acinetobacter calcoaceticus strain EBF65/65 harbours a cryptic plasmid, pAV2, which has been shown by electrophoretic separation on agarose gels to have a molecular mass of approximately 13·5 megadaltons (Md). Transfer of the previously described sex factor pAV1 (Hinchliffe & Vivian, 1980*a, b*) from the hospital strain JC17 into strains possessing pAV2 occurs only at a low frequency, whereas transfer to similar strains lacking pAV2 occurs at a much higher frequency. In EBF65/65, pAV1 may be present in strains possessing or lacking pAV2; pAV1 strains lacking pAV2 correspond to strains previously described as pAV1*a* (Hinchliffe & Vivian, 1980*b*) whereas pAV1 strains which also possess pAV2 correspond to pAV1*b* strains. The genetic evidence presented here is consistent with the hypothesis that pAV2 specifies a host restriction and modification system that is active against pAV1. Physical evidence from agarose gel electrophoresis indicates that pAV1 corresponds to a band of approximately 85 Md in strain JC17. The corresponding band in strains of EBF65/65 is difficult to distinguish because of the presence of a further cryptic plasmid band of approximately 88 Md, designated pAV3. A small cryptic plasmid of approximately 6 Md, designated pAV4, is reported for EBF65/65.

INTRODUCTION

Hinchliffe & Vivian (1980*a*) reported the discovery of a naturally occurring plasmid pAV1, which was transmissible within genetically marked derivatives of *Acinetobacter calcoaceticus* strain EBF65/65. Plasmid pAV1 was discovered in a hospital strain of *A. calcoaceticus* and mediates resistance to sulphonamides. Subsequently, Hinchliffe & Vivian (1980*b*) found that pAV1 shows two distinct levels of transfer within EBF65/65 and concluded that pAV1 can exist in two alternative states, arbitrarily designated *a* and *b*. pAV1*a* strains showed low frequencies of sex factor transfer to recipient strains of EBF65/65, whereas pAV1*b* strains showed high frequencies of transfer.

In this paper we report the discovery in strain EBF65/65 of a second plasmid, designated pAV2, which affects the frequency of pAV1 transfer. The genetic evidence is consistent with the notion that pAV2 specifies DNA modification and restriction functions. Physical evidence is presented concerning the nature of pAV2, and also of two further cryptic plasmids present in EBF65/65 (designated pAV3 and pAV4).

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METHODS

Acinetobacter strains. These are listed in Table 1, and the derivation and plasmid status of strains is shown in Fig. 1. pAV1a and pAV1b strains were obtained by crossing strains C4141 and C4145, respectively, with other strains of EBF65/65, and screening the sulphonamide-resistant transconjugants for their sensitivity to tetracycline and neomycin. The donor type of these new strains was then checked by mating with other derivatives of EBF65/65. In this way strain C4152 was obtained from a strain with donor characteristics of C4145 and strain C4153 was obtained from strain C4141. Stability experiments of the type described by Hinchliffe & Vivian (1980a) were carried out on these new pAV1 donor strains, and colonies sensitive to sulphonamides were isolated to form strains presumptively cured of pAV1.

Media. Minimal medium and Tryptone Soya Agar (Towner & Vivian, 1976a) were used.

Antibiotics. These were obtained from Sigma and were added to media as freshly prepared solutions at the following final concentrations: sulphadiazine, 500 µg ml⁻¹; rifampicin, 100 µg ml⁻¹.

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

Replica plate mating. The method described by Hinchliffe & Vivian (1980a) was used to determine the inheritance of donor activity amongst pAV1 transconjugants, and to isolate spontaneous 'high frequency' recipient strains of pAV1a.

Stability of pAV1. This was determined as described by Towner & Vivian (1976b).

Agarose gel electrophoresis. Single colonies were lysed directly on agarose gels using the method of Eckhardt (1978). Vertical slab gel electrophoresis was as described by Datta *et al.* (1979). The plasmids used for calibration were in *Escherichia coli* K12 strain J53-1 (*pro met nal*) and are listed in Table 2.

RESULTS

Genetic evidence for pAV2

Frequency of pAV1 transfer. Using various pAV1 donors, crosses were performed with strains which had lost pAV1 on the basis of their sensitivity to sulphonamides (see Fig. 1). Selection for pAV1 transfer was made on the basis of resistance to sulphonamides; the results are presented in Table 3. These show that pAV1b always transferred with a high frequency ($> 3 \times 10^{-3}$ transconjugants per recipient) into all the recipients tested, whereas pAV1a shows two distinct levels of transfer depending on the recipient strain used. For example, recipient strains C4159 and C4164 cured of pAV1a, act as 'high frequency' recipients of pAV1a, whereas strains C4157 and C4162, cured of pAV1b, act as 'low frequency' recipients of pAV1a, as do strains such as C426 which have never harboured pAV1. These results show that strains cured of pAV1a do not distinguish the two frequencies of pAV1 transfer when used as recipients in crosses, whereas other strains do.

Inheritance of donor activity. Table 3 also shows the inheritance of donor activity amongst transconjugants from crosses involving pAV1 transfer. Recipient strains which were unable to distinguish the different frequencies of pAV1 transfer, such as C4159 and C4164, rarely gave rise to transconjugants of the pAV1b-type. However, other recipient strains, such as C426 and C4157, when crossed with pAV1b donors yielded only pAV1b transconjugants, whereas these same recipients when crossed with pAV1a donors yielded both pAV1a and pAV1b transconjugants.

Isolation of 'high frequency' recipient strains. The results presented above suggested a possible means of deriving recipient strains spontaneously which could no longer distinguish the two frequencies of pAV1 transfer. Using the replica plate mating technique, colonies of strain C484 were screened for their ability to receive pAV1 from a lawn of C4153. On replica plating to selective media one colony showed a level of receipt of sulphonamide resistance much above that of other colonies. This colony was isolated to form strain C4161, which was later shown to behave like strains cured of pAV1a in respect of its ability to receive pAV1. By a similar procedure strains C4169 and C4177 were isolated (see Fig. 1). The frequency at which these 'high frequency' recipient strains were isolated was approximately 1 in 1000. Thus, we were able to derive spontaneously recipients which could receive

Table 1. *Strains of Acinetobacter calcoaceticus*

Strain	Relevant characteristics*	Reference/source
JC17	TC NM (pAV1)	Hinchliffe & Vivian (1980a)
EBF65/65		Towner & Vivian (1976a)
C48	<i>met-1 ile-1</i>	Auxotroph of EBF65/65
C426	<i>trp-2 his-1</i>	Auxotroph of EBF65/65
C484	<i>phe-1 thi-2</i>	Auxotroph of EBF65/65
C4117	<i>trp-2 his-1</i> (pAV1b)	This paper
C4121	<i>trp-2 his-1 rif-1</i>	Auxotroph of EBF65/65
C4141	<i>trp-2 his-1 rif-1</i> TC NM (pAV1a)	Hinchliffe & Vivian (1980b)
C4145	<i>trp-2 his-1 rif-1</i> TC NM (pAV1b)	Hinchliffe & Vivian (1980b)
C4151	<i>trp-2 his-1</i> (pAV1b)	Hinchliffe & Vivian (1980b)
C4152	<i>met-1 ile-1</i> (pAV1b)	Hinchliffe & Vivian (1980b)
C4153	<i>met-1 ile-1</i> (pAV1a)	Hinchliffe & Vivian (1980b)
C4157	<i>met-1 ile-1</i>	C4152 Su-sensitive†
C4159	<i>met-1 ile-1</i>	C4153 Su-sensitive
C4161	<i>phe-1 thi-2</i>	This paper
C4162	<i>trp-2 his-1</i>	C4151 Su-sensitive
C4163	<i>trp-2 his-1</i> (pAV1a)	C4153 × C426
C4164	<i>trp-2 his-1</i>	C4163 Su-sensitive
C4169	<i>trp-2 his-1</i>	This paper
C4175	<i>phe-1 thi-2 rif-5</i>	C4161 <i>rif-5</i>
C4177	<i>trp-2 his-1</i>	This paper
C4179	<i>phe-1 thi-2 rif-5</i> (pAV1a)	This paper

* TC and NM indicate resistances to tetracycline and neomycin, respectively, which are probably plasmid-borne.

† Su, sulphonamides.

Fig. 1. Derivation of strains of *A. calcoaceticus* carrying plasmids pAV1 and pAV2. TC and NM correspond to resistances that are probably plasmid-borne; × indicate matings between strains; ➔ indicate variant strains derived by spontaneous plasmid loss, except strain C4175 which was derived by spontaneous mutation.

Table 2. *Calibration plasmids used for single colony lysates*

Plasmid	Molecular mass (Md)	Reference
R300B	5.7	Jacob <i>et al.</i> (1977)
R7K	20	Jacob <i>et al.</i> (1977)
RP4	36	Jacob <i>et al.</i> (1977)
R702	46	Jacob <i>et al.</i> (1977)
R1	62	Jacob <i>et al.</i> (1977)
RAI-1	85	Jacob <i>et al.</i> (1977)

Table 3. *Frequency of pAV1 transfer and inheritance of donor activity*

Donor	Recipient	Frequency of pAV1 transfer*	Inheritance of pAV1-type†		No. of transconjugants tested
			pAV1a	pAV1b	
C4152(pAV1b)	C426	High	0	704	704
	C4162(pAV1b ⁻)	High	0	50	50
	C4164(pAV1a ⁻)	High	150	0	150
	C4161	High	231	2	233
	C4169	High	149	1	150
C4151(pAV1b)	C4157(pAV1b ⁻)	High	0	160	160
	C4159(pAV1a ⁻)	High	150	0	150
	C4161	High	151	0	151
C4153(pAV1a)	C426	Low	46	19	65
	C4162(pAV1b ⁻)	Low	71	107	178
	C4164(pAV1a ⁻)	High	48	0	48
	C4177	High	ND	ND	ND
	C4161	High	147	0	147
	C4169	High	100	0	100
C4163(pAV1a)	C4157(pAV1b ⁻)	Low	77	42	119
	C4159(pAV1a ⁻)	High	150	0	150
	C4161	High	153	0	153
JC17(pAV1)	C4175	High	150	0	150

ND, Not determined.

* The frequency of pAV1 transfer was designated as high or low, where high indicates a frequency greater than 3×10^{-3} pAV1 transconjugants per recipient, and low indicates a frequency less than 3×10^{-5} pAV1 transconjugants per recipient.

† The inheritance of pAV1-type was determined by replica plate mating of sulphonamide-resistant transconjugants with appropriately marked auxotrophs of EBF65/65.

pAV1 of both types at a high frequency; none of these recipient strains had previously experienced pAV1.

In view of the results obtained in Table 3 an attempt was made to select for transfer of pAV1 directly from strain JC17 into a 'high frequency' recipient strain, C4175 (see Fig. 1), on the basis of resistance to sulphonamides. In this cross, 2.9×10^{-2} pAV1 transconjugants per recipient were obtained. A sample of 150 transconjugants were all of the pAV1a type with respect to their donor ability (Table 3). In a similar cross between JC17 and C4121, Hinchliffe & Vivian (1980*a*) failed to obtain pAV1 transconjugants.

The genetic results presented above are consistent with the hypothesis that strain EBF65/65 harbours a plasmid, designated pAV2, which is capable of influencing the frequency of pAV1 transfer. pAV2 can be lost spontaneously from strains of EBF65/65, so generating 'high frequency' recipient strains.

Fig. 2. Analysis of single colony lysates of *A. calcoaceticus* by agarose gel electrophoresis. Lysates were prepared as described by Eckhardt (1978) using a 0.7% vertical agarose slab gel. Electrophoresis was for 4 h at a constant 170 V.

Tracks 1 to 5 were *E. coli* K12 J53-1 strains containing the calibration plasmids described in Table 2. Tracks 6 to 11 were *A. calcoaceticus* strains described in Table 1: (1) J53-1(R7K + R300B); (2) J53-1(RP4); (3) J53-1(R702); (4) J53-1(R1); (5) J53-1(RAI-1); (6) C4161; (7) C4162; (8) C4163; (9) C4164; (10) JC17; (11) C426.

Fig. 3. Analysis of single colony lysates of *A. calcoaceticus* by agarose gel electrophoresis. Lysates were prepared as described by Eckhardt (1978) using a 0.7% vertical agarose slab gel. Electrophoresis was for 4 h at a constant 150 V.

Tracks 1 to 4 were *E. coli* K12 J53-1 strains containing the calibration plasmids described in Table 2. Tracks 5 to 8 were *A. calcoaceticus* strains described in Table 1: (1) J53-1(RP4); (2) J53-1(R702); (3) J53-1(R1); (4) J53-1(RAI-1); (5) C426; (6) C4152; (7) C4157; (8) JC17.

Molecular evidence for pAV2

To determine the molecular nature of pAV2, strains C426, C4161, C4162, C4163 and C4164 were examined using the single colony lysis method of Eckhardt (1978). Agarose gel electrophoresis (Fig. 2) revealed that strains C426 and C4162 (tracks 11 and 7) had a plasmid band that was not present in the other strains. The approximate mass of this plasmid, determined using the calibration plasmids (Table 2) in tracks 1 to 5, was 13.5 megadaltons (Md). We have also seen this 13.5 Md plasmid band in strains C4152 and C4157 (Fig. 3, tracks 6 and 7) and C48, C484, C4145 and C4151 (E. Hinchliffe & A. Vivian, unpublished results). Strain C426 also showed two other plasmid bands as well as pAV2 which we arbitrarily designate pAV3 (Fig. 2, track 11; Fig. 3, track 5) and pAV4 (Fig. 2, track 11). The masses of these plasmids are approximately 88 and 6 Md, respectively, and they appear to be present in all the strains of EBF65/65 so far examined. Additionally, there appear to be very faint bands migrating between pAV3 and pAV2 in strain C426 and between the chromosome and pAV4 in strain C4161 (Fig. 2, track 6); these are thought to be artefacts.

Strain JC17 (Fig. 2, track 10; Fig. 3, track 8) has a very clear plasmid band of approximately 30 Md and a faint band of approximately 85 Md. There appears to be a double band in the region of pAV3 in strain C4152 (Fig. 3, track 6); the second band is absent in strain C4157 (Fig. 3, track 7) which has been cured of pAV1. It is therefore suggested that the smaller of the two plasmids is pAV1, which migrates with a band of approximately 85 Md in strain JC17; this double band feature has also been seen in strains C4151, C4153 and C4163 on a number of separate occasions. It should be noted in this context that it is often difficult to visualize pAV1 in both JC17 and strain EBF65/65; the similarity in size between pAV1 and pAV3 also makes it difficult to distinguish the two bands in pAV1-containing strains of EBF65/65 (see strain C4163, Fig. 2, track 8).

The band of approximately 30 Md in strain JC17 mentioned above probably corresponds to a single plasmid specifying resistance to tetracycline and neomycin; the molecular nature of this plasmid is currently under investigation.

DISCUSSION

The physical data presented in this paper unambiguously show the presence of a 13.5 Md plasmid, pAV2, which is present in some strains of EBF65/65 and absent in others (Figs. 1 and 2). Considering the genetic results (Table 3) in the light of the molecular evidence it is apparent that pAV2 is responsible for the variation in the fertility of the sex factor pAV1. pAV1a donor strains C4153 and C4163 lack pAV2 and transfer sulphonamide resistance at a low frequency to strains possessing pAV2, for example C426, C4157 and C4162. However, strains C4159, C4164, C4161, C4169 and C4177 which lack pAV2 receive sulphonamide resistance with a 'high frequency'. This may appear to be a phenomenon of entry exclusion, where pAV2 in the recipient inhibits the entry of pAV1a (Novick, 1969). However, when one considers crosses involving pAV1b donors (i.e. possessing pAV2) with pAV2 recipients there is no apparent exclusion of pAV1. We conclude from this that pAV2 is able to restrict the entry of pAV1a but not pAV1b. This observation is consistent with the notion that pAV2 is able to mediate pAV1 DNA restriction and modification (Arber, 1974) rather than entry exclusion.

Thus, pAV1 in strains C4152 and C4151 has been modified and in consequence co-exists in these strains with pAV2. When this modified pAV1 is transferred to strains C426, C4157 and C4162, which also possess pAV2, there is a high frequency of pAV1 transfer. Strains C4153 and C4163 do not contain a modified pAV1 and therefore when pAV1 is transferred to strains C426, C4162 and C4157 there is restriction of pAV1 and an apparent low frequency of pAV1 transfer.

Most authors who have reported the occurrence of plasmids able to mediate DNA restriction and modification report activity against bacteriophage DNA (Watanabe *et al.*, 1964, 1966; Bannister & Glover, 1968; Jacoby & Sutton, 1977). We are unable to demonstrate restriction of phage DNA by pAV2, since we lack phage for *A. calcoaceticus* EBF65/65. Consequently, we believe our observations are consistent with those of Arber & Morse (1965) and Arber & Wauters-Willems (1970) who report plasmid-mediated restriction affecting the acceptability of plasmid DNA and bacterial conjugation.

Accepting that pAV2 is able to restrict pAV1 we are now able to explain a number of our genetic observations in terms of pAV2. The derivation of 'high frequency' recipients of pAV1a in strains C484, C426 and C4162 is indicative of the loss of pAV2 in EBF65/65. The results presented here suggest that pAV2 is lost spontaneously at a frequency of approximately 1 in 1000. Similarly, the inheritance of pAV1 donor activity (Table 3) can be explained in terms of the presence or absence of pAV2 in the resulting transconjugants. It can be seen from Table 3 that all pAV1b donors produce pAV1b transconjugants in crosses with pAV2 recipients; however, pAV1a donors produce both a- and b-type transconjugants in matings with the same pAV2 recipients. The largest proportion of transconjugants in the latter type of cross inherit a-type characteristics; this may be a result of pAV1 selectively transferring into those cells which have lost pAV2 by instability and which no longer restrict pAV1 entry. Alternatively, there may be active displacement of pAV2 by pAV1, though if this were the case then one might expect to obtain a proportion of pAV1a transconjugants in matings between pAV1b donors and pAV2 recipients and this is not apparent. The fact that pAV1a donors when crossed with a pAV2 recipient give rise to pAV1b transconjugants implies that pAV1 is not always restricted on entry but occasionally escapes restriction and becomes modified. This shows that the modifying action of pAV2 may compete with its restricting ability, protecting a certain proportion of the infecting unmodified pAV1 DNA (Arber, 1974). When pAV1b donors were crossed with recipients C4161, C4164 and C4169 which lack pAV2, nearly all of the resulting transconjugants were pAV1a-type. This is what one would expect if pAV2 modifies pAV1b, since pAV1 transconjugants which lack pAV2 will no longer be modified. However, approximately 1 in 100 to 150 pAV1 transconjugants show pAV1b donor activity. This can be explained in terms of pAV2 mobilization mediated by pAV1, in a similar way to that described for the non-transmissible resistance determinants for tetracycline and neomycin reported previously (Hinchliffe & Vivian, 1980*b*). The fact that pAV1 is able to mediate pAV2 transfer is a most interesting phenomenon, since one might expect that receipt of pAV2 by strains which previously lacked pAV2 would be lethal, especially if EBF65/65 chromosomal DNA is sensitive to pAV2-mediated restriction. The fact that one can derive pAV2 transconjugants in this way suggests that pAV2 must, at least in some cases, preferentially modify on entry into a new recipient as opposed to restrict.

The transfer of pAV1 from strain JC17 into a recipient strain lacking pAV2 resembles pAV1a transfer to recipients lacking pAV2 within strain EBF65/65. This confirms the previous speculation (Hinchliffe & Vivian, 1980*b*) that pAV1 exists in the a-type form in the parent strain JC17, a form which we can now describe as unmodified.

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