Plasmid Transfer and Behaviour in *Acinetobacter calcoaceticus* EBF65/65

By B. A. CHOPADE, P. J. WISE† AND K. J. TOWNER*

Department of Microbiology and PHLS Laboratory, University Hospital,
Queen's Medical Centre, Nottingham NG7 2UH, UK

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At least one plasmid from each of the incompatibility groups B, C, F_{IV}, H₂/S, I α , I δ , P, W and X was shown to be capable of transfer from *Escherichia coli* K12 to *Acinetobacter calcoaceticus* EBF65/65. Transfer was influenced by the presence of pAV2 (thought to encode a restriction-modification system) in the recipient strain; however, not all plasmids belonging to a particular incompatibility group behaved identically. All plasmids were unstable to varying degrees in *A. calcoaceticus* EBF65/65, but under suitable conditions were capable of transfer to further strains of EBF65/65 and re-transfer to *E. coli* K12. Of 40 recently isolated trimethoprim R plasmids 31 transferred successfully from *E. coli* K12 to *A. calcoaceticus* EBF65/65, but 17 of these 31 required the introduction of a second mobilizing plasmid for re-transfer to occur.

INTRODUCTION

There is currently great interest in the possible role of *Acinetobacter calcoaceticus* as a reservoir of antibiotic resistance genes in hospitals. Apart from their occasional pathogenic importance (e.g. Glew *et al.*, 1977; Retailliau *et al.*, 1979), strains of *Acinetobacter* can be isolated from environmental sources such as water-baths, room humidifiers, respiratory equipment and disinfectants (French *et al.*, 1980). They also occur on human skin, where they favour moist regions such as toe-webs, axillae and the groin (Noble & Pitcher, 1978; Al-Khoja & Darrell, 1979), and can be isolated from conjunctivae and the respiratory and genito-urinary tracts (Buxton *et al.*, 1978; Gilardi, 1978). Many strains of *Acinetobacter* are resistant to a variety of clinically important antibiotics (e.g. French *et al.*, 1980; Devaud *et al.*, 1982; Goldstein *et al.*, 1983) and it has been demonstrated that even in cases where resistance genes are not initially plasmid-mediated, transposition of such genes from the chromosome to a suitable vector plasmid can occur, leading to their potential dissemination in more important pathogens (Devaud *et al.*, 1982).

Members of the P incompatibility group of plasmids were initially thought to be the only plasmids capable of transfer from *Escherichia coli* K12 to *A. calcoaceticus* (Towner & Vivian, 1977). Subsequently it was demonstrated that the recipient strain of *A. calcoaceticus* used (EBF65/65) contained a plasmid, pAV2, which is responsible for variation in the fertility of plasmids and may determine a restriction-modification system in its host strain (Hinchliffe *et al.*, 1980). Using a pAV2⁻ strain as a recipient, Hinchliffe & Vivian (1980*b*) were able to demonstrate transfer of the W group plasmids R388 and S-a into EBF65/65 from *E. coli* K12; however, transfer of plasmids belonging to the C, F_{II} or N incompatibility groups was not observed. In contrast, other workers have been able to demonstrate the transfer of a group C plasmid from a clinical strain of *A. calcoaceticus* to *E. coli* K12 (Goldstein *et al.*, 1983).

In an attempt to resolve this and other anomalies, we report here the results of a re-evaluation of the ability of plasmids from 17 different incompatibility groups to transfer resistance genes to and from *A. calcoaceticus*. Results obtained with two sets of plasmids are reported: firstly, a set

† Present address: Department of Pure and Applied Biology, Imperial College, London SW7 2BB, UK.

of 'standard' plasmids which are well-known and are often regarded as being representative of their particular incompatibility groups: secondly, in order to test the wider validity of the results obtained, a set of 40 trimethoprim R plasmids belonging to different incompatibility groups which were recently isolated from bacteria in the Nottingham area (Towner & Wise, 1983). In addition, we have studied the possibility that non-conjugative plasmids can be mobilized to and from *A. calcoaceticus*, together with the subsequent behaviour of such plasmids in this host.

METHODS

Bacterial strains and plasmids. These are listed in Table 1.

Media. *Acinetobacter* minimal medium was as described by Towner & Vivian (1976*a*). *E. coli* minimal medium was that of Vogel & Bonner (1956). Supplements were stored as sterile solutions and were added to minimal media immediately before use at the following final concentrations (mg l⁻¹): individual amino acids, 50; individual vitamins, 1. Complete media were Oxoid DST agar and Oxoid Nutrient Broth (NB).

Antibiotics. These were from standard commercial sources and were used at the following final concentrations (mg l⁻¹): sodium ampicillin, 100; chloramphenicol, 25; tetracycline hydrochloride, 5; trimethoprim lactate, 100.

Plasmid analysis. The filter mating method of Towner & Vivian (1976*b*) was used for all crosses, with transfer frequencies being expressed in terms of the number of transconjugants per recipient cell. Where necessary, tests for the co-transfer of resistance markers other than that selected were made using antibiotic discs (Mast Ltd). Transposition of trimethoprim resistance genes to RP4 was detected following re-transfer of RP4 to *E. coli* K12 as previously described (Towner, 1981). Plasmids were physically analysed in *E. coli* K12 by agarose gel electrophoresis (Silhavy *et al.*, 1984).

Stability of plasmids. The stability of different plasmids in *A. calcoaceticus* EBF65/65 was tested under two sets of conditions. (i) A single colony of a plasmid-carrying strain, grown on a DST plate containing an appropriate selective antibiotic, was used to inoculate 10 ml NB containing the same antibiotic in a 100 ml conical flask. This was vigorously aerated at 28 °C on a rotary shaker until the culture reached a density corresponding to approx. 5×10^8 cells ml⁻¹ (about 6 to 7 h). This represented about 12 generations of growth. Portions (0.1 ml) of appropriate dilutions (in quarter-strength Ringer's solution) of this culture were then spread on DST plates with and without added antibiotic. Following incubation (48 to 72 h, 28 °C), the number of cells per ml capable of forming colonies on each medium set was calculated. (ii) A 1 ml portion of the culture grown in the presence of antibiotic was then used to inoculate 9 ml of fresh antibiotic-free NB and the same procedure as above followed. Plasmid stability was expressed as the number of cells subsequently capable of forming colonies on antibiotic-containing medium divided by the total viable count on antibiotic-free medium.

Table 1. *Bacterial strains and plasmids*

Bacterial strains

Acinetobacter calcoaceticus EBF65/65 (Hinchliffe *et al.*, 1980)

C48 *ile-1 met-1* (pAV2)

C426 *trp-2 his-1* (pAV2)

C499 Rif^r mutant of C48

C4161 *phe-1 thi-2*

C4169 *trp-2 his-1*

Escherichia coli K12

J53.2 F⁻ *pro met* Rif^r (Towner, 1980)

Standard representative plasmids (Jacob *et al.*, 1977)

RA1 (IncA/C); R724 (IncB); R57b (IncC); R386 (IncF_I); R136 (IncF_{II}); R124 (IncF_{IV}); R27 (IncH_I); R478 (IncH₂/S); R64 (IncI_α); R821a (IncI_δ); R621a (IncI_γ); R387 (IncK); R446b (IncL/M); N3T (IncN); RP4 (IncP); R751 (IncP); R388 (IncW); R6K (IncX).

Recently isolated trimethoprim R plasmids (Towner & Wise, 1983)

IncB: pUN65, pUN161, pUN165, pUN193, pUN259, pUN345, pUN365, pUN422, pUN548.
IncF_{II}: pUN166, pUN210, pUN237, pUN241, pUN261, pUN334, pUN420, pUN494, pUN510.
IncI_α: pUN202, pUN283, pUN394, pUN506.
IncI_δ: pUN174, pUN215, pUN253, pUN349, pUN363, pUN382, pUN439, pUN549, pUN583.
IncK: pUN333.
IncM: pUN550.
IncP: pUN108, pUN189, pUN240, pUN373.
IncW: pUN191.
IncX: pUN300, pUN428.

Table 2. Transfer of standard representative plasmids from *E. coli* K12 to *A. calcoaceticus* EBF65/65

Matings were done overnight at 28 °C on filters as described in Methods. Frequencies of transfer are expressed in terms of transconjugants per recipient cell and are the mean of at least two determinations.

Donor	Inc group	Recipient	Selection*	Frequency of transfer
J53.2(R57b)	C	C4169	Cm	1.1×10^{-3}
J53.2(R57b)	C	C426	Cm	3.7×10^{-5}
J53.2(R124)	F _{IV}	C4169	Tc	1.0×10^{-5}
J53.2(R124)	F _{IV}	C426	Tc	$< 1.6 \times 10^{-9}$
J53.2(R478)	H ₂ /S	C4169	Tc	4.1×10^{-4}
J53.2(R478)	H ₂ /S	C426	Tc	9.1×10^{-8}
J53.2(R64)	I α	C4169	Tc	8.5×10^{-6}
J53.2(R64)	I α	C426	Tc	1.6×10^{-6}
J53.2(R821a)	I δ	C4169	Ap	2.6×10^{-5}
J53.2(R821a)	I δ	C426	Ap	2.3×10^{-6}
J53.2(RP4)	P	C4169	Tc	6.4×10^{-4}
J53.2(RP4)	P	C426	Tc	9.2×10^{-7}
J53.2(R388)	W	C4169	Tp	2.7×10^{-7}
J53.2(R388)	W	C426	Tp	$< 1.2 \times 10^{-9}$
J53.2(R6K)	X	C4169	Ap	9.5×10^{-5}
J53.2(R6K)	X	C426	Ap	6.3×10^{-5}

* Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; Tp, trimethoprim.

RESULTS

Attempted transfer of 'standard' plasmids to *A. calcoaceticus*

Strains of *E. coli* K12 J53.2 carrying the representative 'standard' plasmids listed in Table 1 were crossed with *A. calcoaceticus* EBF65/65. No transconjugants were obtained when plasmids representing incompatibility groups B, F_I, F_{II}, H₁, I γ , K, M or N were crossed with either C4169 or C426 ($< 1 \times 10^{-9}$). In contrast, antibiotic resistance markers conferred by plasmids representing incompatibility groups C, F_{IV}, H₂/S, I α , I δ , P, W and X transferred successfully to C4169. In the cases where it was possible to test for the co-transfer of markers other than that used for selection purposes, these had also been successfully transferred. With the exceptions of R124 and R388 (the F_{IV} and W group representatives), transfer also occurred to the isogenic pAV2⁺ strain C426, generally at a reduced frequency, although the influence of pAV2 was considerably more apparent with some plasmids than with others (Table 2). There was some evidence to suggest that not all of the plasmid resistance genes were expressed equally well in EBF65/65. For example, RP4 transconjugants selected on tetracycline generally appeared following 36 h incubation at 28 °C, whereas incubation for 72 to 96 h was required for R124 (no colonies were observed on control plates).

In the cases where plasmid transfer was not detected to either C4169 or C426, the P group plasmid R751 was introduced into the original donor strains of J53.2 for use as a potential mobilizing vector. When the resulting R751 transconjugants were crossed with C4169, transfer of R751 occurred at frequencies $> 1 \times 10^{-5}$, but no mobilization of the original resident plasmid was detected ($< 1 \times 10^{-9}$).

Stability of 'standard' plasmids in *A. calcoaceticus* EBF65/65

It was observed at an early stage that all eight of the representative plasmids successfully transferred were unstable to varying degrees in *A. calcoaceticus* EBF65/65. In each case it was found that stable maintenance required continual sub-culture on media containing appropriate selective antibiotics. Table 3 presents the results of plasmid stability tests on cultures grown in the presence or absence of a selective antibiotic as described in Methods.

Table 3. *Plasmid stability tests in A. calcoaceticus EBF65/65*

Derivatives of strain C4169 carrying the different plasmids were grown in (i) antibiotic-containing broth and (ii) antibiotic-free broth. Plasmid stability is expressed as the number of cells subsequently capable of forming colonies on antibiotic-containing agar divided by the total viable count on antibiotic-free agar. Results shown are the mean of at least two determinations. Antibiotic abbreviations are as for Table 2.

Plasmid	Selection	Plasmid stability after growth in:	
		Antibiotic-containing broth	Antibiotic-free broth
R57b	Cm	0.81	0.17
R124	Tc	0.99	0.95
R478	Tc	0.77	0.80
R64	Tc	0.96	0.94
R821a	Ap	0.67	0.36
RP4	Tc	0.87	0.80
R388	Tp	0.75	0.18
R6K	Ap	0.30	0.30

Table 4. *Ability of A. calcoaceticus C4169 transconjugants to act as subsequent donors*

Matings were done overnight at 28 °C on filters as described in Methods. In some cases (denoted *), donor cells were grown in the presence of selective antibiotic following initial failure to detect transfer. Frequencies of transfer are expressed in terms of transconjugants per recipient cell and are the mean of at least two determinations. Antibiotic abbreviations are as for Table 2.

Donor	Recipient	Selection	Transfer frequency
C4169(R57b)	C4161	Cm	4.7×10^{-2} *
	C499	Cm	1.6×10^{-3} *
	J53.2	Cm	6.5×10^{-3} *
C4169(R124)	C4161	Tc	$< 7.0 \times 10^{-8}$ *
	C499	Tc	$< 1.6 \times 10^{-9}$ *
	J53.2	Tc	7.2×10^{-6}
C4169(R478)	C4161	Tc	4.0×10^{-7} *
	C499	Tc	$< 1.6 \times 10^{-9}$ *
	J53.2	Tc	3.8×10^{-5}
C4169(R64)	C4161	Tc	$< 6.3 \times 10^{-9}$ *
	C499	Tc	$< 1.1 \times 10^{-9}$ *
	J53.2	Tc	1.3×10^{-4}
C4169(R821a)	C4161	Ap	1.5×10^{-5}
	C499	Ap	2.2×10^{-5}
	J53.2	Ap	4.9×10^{-6}
C4169(RP4)	C4161	Tc	8.1×10^{-1}
	C499	Tc	2.7×10^{-1}
	J53.2	Tc	7.6×10^{-2}
C4169(R388)	C4161	Tp	1.6×10^{-4}
	C499	Tp	1.0×10^{-6}
	J53.2	Tp	1.5×10^{-3}
C4169(R6K)	C4161	Ap	5.3×10^{-4}
	C499	Ap	2.0×10^{-7}
	J53.2	Ap	1.9×10^{-2}

Re-transfer of 'standard' plasmids from EBF65/65

The ability of purified C4169 transconjugants to act as donors in subsequent transfer experiments with either pAV2⁻ or pAV2⁺ strains of EBF65/65 or strains of *E. coli* K12 as recipients is shown in Table 4. All of the C4169 transconjugants were capable of acting as

plasmid donors to *E. coli* K12; however, in certain cases (Table 4) transfer was only detected when the donor strain was grown in the presence of a selective antibiotic, perhaps reflecting the inherent instability of certain plasmids in EBF65/65 described above. Plasmids transferred back to *E. coli* K12 retained all of their original resistance markers. The probable influence of pAV2 was again apparent in crosses between strains of EBF65/65 with most plasmids transferring at a reduced frequency from C4169 (pAV2⁻) to C499 (pAV2⁺) compared to C4161 (pAV2⁻). Interestingly, transfer of R124 and R64 was not detected from C4169 to either C499 or C4161 although they transferred successfully in an apparently unaltered form to J53.2. R124 and R64 behaved as extrachromosomal elements when crossed with *E. coli* K12 and, at present, we have no explanation for this repeated observation which is the subject of further investigation (see Discussion).

Attempted transfer of recently isolated plasmids

There is some evidence (Towner, 1980) to suggest the presence in EBF65/65 of a second restriction system, independent from that coded for by pAV2, which could affect the transfer of certain plasmids to pAV2⁻ strains. It was considered possible that, even within a single incompatibility group, there could be variations as to whether or not plasmids would transfer to EBF65/65, dependent upon the availability and number of suitable restriction sites. To test this possibility (and the wider validity of the results obtained with the 'standard' plasmids), strains of *E. coli* K12 carrying the 40 different recently isolated trimethoprim R plasmids listed in Table 1 were crossed with C4169 in the usual manner, with selection being made for the transfer of trimethoprim resistance. Where necessary, mobilization experiments (with RP4 as the mobilizing vector) and back-crosses from C4169 to *E. coli* K12 were also attempted. No attempt is made in this paper to describe in detail the complex patterns and varied frequencies of plasmid transfer obtained; however, it is possible to summarize the properties and behaviour of the 40 plasmids tested by dividing them into the following five distinct groups.

Group 1 comprising four IncP plasmids (pUN108, pUN189, pUN240, pUN373), two IncB plasmids (pUN161, pUN345) and one IncW plasmid (pUN191). These seven plasmids were transferred to C4169 and were subsequently able to re-transfer back to *E. coli* K12.

Group 2 comprising three IncB plasmids (pUN65, pUN193, pUN365) and one IncX plasmid (pUN300). These four plasmids transferred to C4169, but subsequent re-transfer to *E. coli* K12 was not detected. When RP4 was introduced into the C4169 transconjugants, three of the four plasmids (pUN65, pUN365 and pUN300) were mobilized by RP4 to *E. coli* K12, but retained their identity as separate replicons. In contrast, mobilization of pUN193 by RP4 to *E. coli* K12 was never detected. None of these four plasmids carried transposable trimethoprim resistance.

Group 3 comprising nine IncF_{II} plasmids (pUN166, pUN210, pUN237, pUN241, pUN261, pUN334, pUN420, pUN494, pUN510), four IncI_α plasmids (pUN202, pUN283, pUN394, pUN506), one IncK plasmid (pUN433) and one IncM plasmid (pUN550). Transfer of these 15 plasmids to C4169 was not detected and the trimethoprim resistance genes were not mobilized by RP4.

Group 4 comprising nine IncI_δ plasmids (pUN174, pUN215, pUN253, pUN349, pUN363, pUN382, pUN439, pUN549, pUN583). Transfer of these nine plasmids to C4169 was not detected, but each plasmid carried transposable trimethoprim resistance. Mobilization experiments using RP4 resulted in the appearance of trimethoprim-resistant transconjugants following prior transposition of the trimethoprim resistance genes onto RP4 in the *E. coli* K12 donor. The original IncI_δ plasmids were not mobilized.

Group 5 comprising four IncB plasmids (pUN165, pUN259, pUN422, pUN548) and one IncX plasmid (pUN428). Transfer of these five plasmids to C4169 was not detected, but each plasmid was capable of being mobilized by RP4. Transfer could occur independently of RP4, but the presence of RP4 was required in the donor strain of *E. coli* K12. Similarly, in back-crosses from C4169 to *E. coli* K12, transfer was not detected unless RP4 was also present in the C4169 donor; however, in each case the trimethoprim R plasmid existed as a separate replicon to RP4. None of these plasmids carried transposable trimethoprim resistance.

DISCUSSION

Combining the results obtained with the representative standard plasmids and the trimethoprim R plasmids that have been more recently isolated, at least one plasmid belonging to each of the following incompatibility groups was capable of transfer to *A. calcoaceticus* EBF65/65 and subsequent re-transfer to *E. coli* K12: groups B, C, F_{IV}, H₂/S, I α , I δ , P, W and X. This represents a considerable increase in the number of plasmid groups which were thought to have this capability, due in part to recognition of the significance of using a suitable restriction-deficient (pAV2⁻) recipient strain and also partly reflecting the fact that not all plasmids belonging to a particular incompatibility group behave identically (perhaps due to the availability and number of particular restriction sites). In this connection it should be noted that the set of representative standard plasmids tested have also been used on a world-wide basis to test the recipient ability of a large variety of different organisms. The results reported in this paper with *A. calcoaceticus* show that tests with a single representative plasmid cannot always be regarded as conclusive.

All of the plasmids transferred to *A. calcoaceticus* were at least partially unstable in the EBF65/65 system. Some of the apparent stability observed could conceivably have resulted from transposition of resistance genes or integration of plasmids into the chromosome: however, the selected markers from plasmids which showed a high degree of stability in antibiotic-free broth are not known to be transposable and all plasmids were capable of re-transfer to *E. coli* K12 in an apparently unaltered form, suggesting that they existed in an extrachromosomal state. The physical analysis of these plasmids in EBF65/65 is complicated by the presence in this host of several resident plasmids of unknown function (Hinchliffe *et al.*, 1980) and it is apparent that EBF65/65 may not be a suitable strain for such physical studies. It was notable that R6K was equally unstable in antibiotic-free broth and ampicillin-containing broth. Possibly there was some protection of cells which had lost R6K by secreted β -lactamase from plasmid-containing cells. A similar effect could possibly account for the relatively low stability observed for R821a in ampicillin-containing broth. The repeated high degree of instability observed with R388 differed from the results obtained by Hinchliffe & Vivian (1980*b*) who used a replica-plate system (Towner & Vivian, 1976*a*) to look for loss of R388-mediated sulphonamide resistance. Tests for sulphonamide resistance are, however, notoriously difficult to do, being dependent both on the medium used and the inoculum size (Garrod *et al.*, 1981); as an alternative, some form of chromosomal integration could also explain apparent stability, although it should be noted in this connection that the sulphonamide resistance gene of R388 is not known to be transposable.

Several anomalous results were found when re-transfer of the plasmids from EBF65/65 to other strains of EBF65/65 was attempted. For example, transfer of RP4 from J53.2 to EBF65/65 was reduced ($\times 10^{-3}$) by the presence of pAV2, but not re-transfer from C4169 to other EBF65/65 strains. In contrast, no obvious effect of pAV2 on the transfer of R6K from J53.2 to EBF65/65 was detected, but an apparent effect was observed when re-transfer was attempted. In a relatively poorly characterized organism such as *A. calcoaceticus* it is difficult to be certain about the reasons for such anomalous results, however, three particular factors could certainly be involved: (i) pAV2 is unstable and is lost from EBF65/65 at a frequency of 1 in 1000 (Hinchliffe *et al.*, 1980), thus in any 'pAV2⁺' cell population a large number of cells will be pAV2⁻; (ii) several other plasmids of unknown function are also present in EBF65/65 (Hinchliffe *et al.*, 1980) and the degree of stability and possible effects of these plasmids on the fertility of introduced plasmids belonging to different incompatibility groups is unknown; (iii) the extent to which secondary rounds of conjugation can occur between initial transconjugants and other recipient cells present on the surface of a filter has not yet been assessed.

It has been suggested that acinetobacters may have an important role as reservoirs or vehicles of transmissible drug resistance in hospitals, where they show widespread and increasing resistance to antibiotics (Vivian *et al.*, 1981). Many clinical isolates contain a variety of different plasmids (e.g. Murray & Moellering, 1979), although these cannot always be directly associated with transferable antibiotic resistance. It has previously been shown that some plasmids in *Acinetobacter* cannot effect their own transfer, but can be mobilized under the influence of a second transmissible plasmid (Hinchliffe & Vivian, 1980*a*; Divers *et al.*, 1984). In this paper, 31

of the 40 trimethoprim R plasmids tested were transmissible from *E. coli* to *A. calcoaceticus*, but 17 of these 31 subsequently required the presence of a second transmissible plasmid for re-transfer (mobilization) of resistance genes to occur. In some cases transposition of resistance genes to the mobilizing plasmid was also observed. In order to clarify the importance of plasmids in antibiotic-resistant clinical isolates of *Acinetobacter* it may therefore be necessary to assess more rigorously the potential for mobilization of resistance genes from such isolates, particularly to potential pathogens other than *E. coli* with which they are likely to be in close contact in the clinical environment.

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